Biochemical Pharmacology

VOLUME 5 1960-1961

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PERGAMON PRESS

Oxford · New York · Paris · Los Angeles

ol.
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960

Vol.
5
1960

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Biochemical Pharmacology, 1960, Vol. 5, pp. 1–19. Pergamon Press Ltd., Printed in Great Britain.

VERSUCHE ZUR ENTDECKUNG NEUER FUNGISTATIKA—I

PHENOL-DERIVATE

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(*Received* 18 *March* 1960)

Abstract—The fungical action of 260 phenol derivatives on a variety of fungi incubated in various liquid and solid media. Information concerning the mode of action of the most active members was obtained by determining the influence of added substances and of pH on the killing action. The fungi were protected by the addition of serum proteins as well as by a variety of lipids. It was concluded that these phenols do not inhibit any one particular biochemical process but have a non-specific denaturing action on the cell wall.

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DIE durch verschiedene menschenpathogene Pilzarten verursachten Krankheiten nehmen eine wichtige Stelle in der Menschenpathologie an und ihr klinisches Vorkommen in der Form von inneren und äusseren Mykosen ist ziemlich verbreitet.

Der Schutz ihnen gegenüber, bzw. die Heilung der entwickelten Krankheiten ist zurzeit noch nicht erfolgreich genug—trotz dem Umstand, dass im Laufe der vorigen Jahrzehnte verhältnismässig umfangreiche Untersuchungen unternommen wurden im Interesse der Entdeckung neuer Fungistatika von ausreichender Wirkung, und dass diesbezügliche Forschungen in unseren Tagen in immer grösseren Umfange geführt werden (siehe die Literatur[1–60] in Bezug auf die fungistatische Wirkung der Phenol-Derivate).

Eben deshalb haben wir entschlossen, die *in vitro* ausgeübte fungistatische Wirkung einer grossen Anzahl von Verbindungen zu untersuchen, und die Abhängigkeit ihrer fungistatischer Wirkung von ihrer chemischen Struktur zu erforschen, und zwar zu dem Zwecke, dass wir die während unserer Untersuchungen erzielten Resultate und Erfahrungen im Laufe unserer folgenden Forschungen auf theoretischer Linie zur Entdeckung weiterer Fungistatika verwenden mögen, andererseits aber im Interesse der klinischen Therapie von Dermatomykosen anwenden können.

Im Laufe unserer Forschungen wurde die fungistatische Wirkung der zu den verschiedensten Gruppen gehörenden Verbindungen (so auch die der Phenol-Derivate) auf menschenpathogene und auch auf apathogene Pilzstämme sowohl auf flüssigem als auch auf festem Nährboden untersucht. Die Mehrzahl der Verbindungen hat Verfasser selbst mittels der in der chemischen Fachliteratur bekannten Methoden synthetisiert; eine kleinere Anzahl derselben stand ihm dagegen als Handelsprodukt von "pro analysi", Reinheitsgrad zur Verfügung.

UNTERSUCHUNGSMETHODE

Für unsere Experimente haben wir das durch Uri und Mitarbeiter[62] für Pilz-Züchtung hervorragend bewährt gehaltene Maische-Kulturmedium gebraucht.

Die Herstellung des Nährbodens erfolgte folgendermassen: Zu 1 kg fein gemahlter gemaischter Gerste wurde 11 l. Brunnenwasser gegeben und an 45 °C Temperatur durch 30 Minuten fortwährend umgerührt. Den Forschritt der Zuckerbildung haben wir durch Jod-Probe kontrolliert; dann wurde die Temperatur sehr langsam, ungefähr während 45 Minuten stufenweise bis 72 °C erhöht. Als die Jod-Probe sich als negativ erwies, wurde das Gemisch nach Bewahren der Temperatur für weitere 15 Minuten noch an 72 °C gründlich gekocht—im Interesse der Vernichtung der Zuckerbildung verursachenden Enzyme—dann filtriert und durch zwei folgende Tage an 100 °C sterilisiert, endlich durch 5 Tage sich setzen gelassen. Danach wurde das Gemisch durch Papiersieb durchgeseiht, auf pH = 6,0–6,2 eingestellt und dann sterilisiert.

Der so gewonnene Maische-Nährboden wurde mit 3% Agar in der Form eines festen "Maische-Agar-Nährbodens" oder nach Zugabe von 56 °C inaktivisierten Rinderserums als "flüssiger Serum-maische-Nährboden" angewandt.

Das pH sowohl des Maische-Agars, wie auch des Serum-Maische-Nährbodens betrug 6,0–6,2.

Die 2%-ige alkoholische oder wässerige Lösung der untersuchten Verbindungen wurde mit Maische-Nährboden auf zehnfaches Volumen verdünnt. Aus den so gewonnenen 0,2%-igen Lösungen, homogenen Suspensionen oder Emulsionen ausgegangen wurden die auf flüssigem Serum-Maische-Nährboden vorgenommenen Versuchen mit den üblichen Serien-verdünnungsmethode durchgeführt. Die untersuchte niedriegste Verdünnung der Verbindungen war 1:5000. Das Inoculum bildete je ein Tropfchen einer Sporen-Suspension, welche aus der zehn-tägigen auf schiefem Maische Agar-Nährboden gewachsenen Kultur der zu den Versuchen gebrauchten Pilzstämme mittels 10 ml steriler physiologischer Kochsalz-Lösung gewonnen wurde.

Die Pilzstämme, welche zu den auf flüssigem Nährboden ausgeführten Versuchen verwendet wurden, waren folgende:

(1) *Penicillium simplicissimum.*
(2) *Aspergillus niger.*
(3) *Trichothecium roseum.*
(4) *Candida albicans.*
(5) *Achorion quinckeanum.*
(6) *Trichophyton gypseum.*
(7) *Epidermophyton Kaufman-Wolff.*

Die Inkubationszeit der Pilzstämme von Nr. 1–4 war 5 Tage, die der Nr. 5–7 aber 10 Tage. Temperatur: 30 °C.

Bei der Auswertung der Ergebnisse haben wir für eine Grenzverdünnung von fungistatischer Wirkung der untersuchten. Verbindungen jene Konzentration genommen, worin nach Verlauf der Inkubationszeit *keine Spur des Wachstums* der Pilze mehr zu erfahren war.

Die in der Tabellen stehenden Ziffern bezeichnen die Reziprokwerte jener Verdünnung der einzelnen Verbindungen, welche zur *vollkommenen Entwicklungshemmung* führen.

Insofern die einzelnen Verbindungen in den im Laufe der Versuchen angewandten niedrigsten Verdünnung (d.h. in 1:5000) nur eine teilweise Entwicklungshemmung herbeiführen konnten, wurde diese hemmende Wirkung noch bezeichnet. Die Art der Bezeichnung in den Tabellen ist: (5000). Eine *teilweise* Entwicklungslähmung, die

sich bei einer grösseren Verdünnung als 1:5000 noch eventuell zeigte, wurde nicht mehr in Betracht genommen, sondern für volles Wachstum angesehen. Wir betrachteten eine Verbindung als unwirksam, wenn sie in der angewandten niedrigsten Verdünnung (also in 1:5000) keine fungistatische Wirkung hat. Wir bezeichneten die Unwirksamkeit der Verbindungen in den Tabellen mit dem folgenden Zeichnen: "—"

Aus jeden Konzentrationen einer jeden Verbindung haben wir innerhalb einer Versuchsserie einzeln drei Parallelen angewandt und jede Versuchsserie dreimal bis viermal reproduziert. Die erzielten Ergebnisse haben sowie bei den einzelnen Parallelen, wie auch bei den zu verschiedenen Gelegenheiten eingestellten identischen Versuchsserien *praktisch eine gute Übereinstimmung* gezeigt—abgerechnet eine geringe Streuung und unbedeutende Abweichungen.

In feste Maische-Agar-Platten von 6,4 mm Dicke wurden Löcher von 10 mm Durchmesser eingebohrt. Danach wurde eine Menge von 0,5 ml der 0,2%-igen Lösung, homogenen Suspension oder Emulsion der untersuchten Verbindungen in die Löcher eingemassen. Diese Lösungen, Suspensionen oder Emulsionen wurden so dargestellt, dass wir eine Menge von 0,5 ml der 2%-igen alkoholishchen oder wässerigen Lösung der Verbindungen zu 4,5 ml aufgeschmolzenem Maische-Agar-Nährboden gaben und gut vermischten.

Die Menge der untersuchten Verbindung in den Löchern der Agarplatte betrug so je 1,0 mg, der Grad ihrer Anfangskonzentration 1:500, welche Konzentration durch die Diffusion in dem umgebenden Nährboden immer mehr abnahm; demgegenüber erhöhte sich die in dem umgebenden Nährboden anwesende Konzentration der Verbindungen auf parallele Weise.

Nach der Erstarrung des "chemikalischen Agars" in den gebohrten Löchern des Nährbodens wurden die Kulturmedien enthaltenden Agar-Platten mit einer Sporen-Suspension eingeimpft, welche aus der zehn-tägigen auf schiefem Maische-Agar-Nährboden gewachsenen Kultur der zu den Versuchen gebrauchten Pilzstämme mittels 10 ml steriler physiologischer Kochsalz-Lösung gewonnen wurde. Diese Sporen-Suspension wurde dann auf der Oberfläche des Nährbodens gleichmässig verteilt.

Die eingeimpften festen Nährboden-Platten wurden dann an 30 °C Temperatur inkubiert und die erzielten Ergebnisse nach 5 Tagen Inkubationszeit abgelesen. Die Auswertung der Resultate erfolgte so, dass wir die Durchmesser der rundförmigen Gebiete, innerhalb welcher das Pilz-Wachstum gehemmt wurden, abgemessen und in mm ausgerechnet haben.

Die Ergebnisse der aur festem Nährboden vorgenommenen Versuchen, wie sie in den Tabellen stehen, sind durch die Ziffern ausgedrückt, welche die Durchmesser dieser wachstumsgehemmte Gebiete, dieser "Auslöschringe" bedeuten, in mm ausgedrückt.

In einer Petri-Schale wurden auf einmal drei bis fünf Verbindungen untersucht (als Ausnahme, bei Versuchen mit Verbindungen von besonders grosser Wirkung nur *eine* auf einmal); innerhalb einer Versuchsserie haben wir zwei Parallelen verwendet und jede Versuchsserie dreimal bis viermal wiederholt.

Die Werte beim Ablesen der einzelnen Parallelen innerhalb einer Serie, sowie jene Werte, die wir bei der wiederholten Einstellung dieselber Versuchsserien erhielten, stimmten miteinander ziemlich gut überein, abgerechnet eine Schwankung von ±10–15%, was bei Untersuchung dieses Typus gar nicht umgewöhnlich zu werten ist.

Die Pilzstämme, die zu den auf festem Nährboden angestellten Versuchen gebraucht wurden, waren folgende:

(1) *Penicillium simplicissimum*.

(2) *Aspergillus niger*.

(3) *Trichothecium roseum*.

(4) *Candida albicans*.

(5) *Saccharomyces cerevisiae*.

Bei der für unsere Untersuchungen angewandten Methode haben sich bloss die verhältnismässig intensiv wirkenden Verbindungen als fungistatisch wirksam bewährt. Diejenige Verbindungen, welche bei Versuchen auf flüssigem Nährboden nicht einmal in der Konzentration von 1:5000 eine gänzlich (oder weinigstens teilweise) hemmende Wirkung auf das Wachstum der Pilze ausüben konnten und bei den Versuchen auf festem Nährboden ein wachstumslosses Gebiet von 10 mm Durchmesser nicht herbeiführen konnten, sich als inaktiv bewährten, und haben wir sie in der Tabellen mit dem Zeichen "—" gemerkt.

Bei der Übersicht der in den Tabellen angegebenen Ergebnisse wird in zahlreichen Fällen wohl auffallen, dass zwischen der Intensität der fungistatischen Wirkung, Welche dieselbe Verbindung auf denselben Pilzstamm einerseits auf flüssigem, andererseits auf festem Nährboden ausgeübt hatte, manchmal jedoch erhebliche Unterschiede vorkommen.

Die Ursache dieses Unterschiedes ist vor allem ein physikalisch-chemisches Problem; die Erörterung dieses Problems gehört nicht zur Zielsetzung dieser Mitteilung.

ERGEBNISSE

Die bei der Untersuchung der fungistatischen Wirkung der Phenol-Derivate erhaltenen Resultate sind in Tabellen 1, 2 und 3 zusammengefasst. Die Zeichenerklärung dieser Tabellen s. bei der Untersuchungsmethode.

Jene von den 260 Phenol-Derivaten der Tabelle 1 und 2, welche in der Tabelle 3 nicht aufgeführt sind, übten auf festem Maische-Agar Nährboden keine fungistatische Wirkung auf die zu den Versuchen verwendeten fünf Pilzstämme aus.

TABELLE 1. DIE FUNGISTATISCHE WIRKUNG VON PHENOL-DERIVATEN AUF FLÜSSIGEM SERUM-MAISCHE NÄHRBODEN

Nr.	Verbindungen	*Penicillium simplicissimum*	*Aspergillus niger*	*Trichothecium roseum*	*Candida albicans*	*Achorion quinckeanum*	*Trichophyton gypseum*	*Epidermophyton Kaufman-Wolff*
	1. Alkyl-, Aryl-, Alkyloxy- und Alkoyl-Phenole							
F/2	2-Methyl-phenol	—	—	—	—	5000	5000	5000
F/3	3-Methyl-phenol	—	—	—	—	5000	5000	5000
F/4	4-Methyl-phenol	—	—	—	—	5000	5000	5000
F/5	2. 4-Dimethyl-phenol	—	—	(5000)	—	5000	5000	5000
F/6	3. 4-Dimethyl-phenol	—	—	5000	—	5000	5000	5000
F/7	2-i-Propyl-5-methyl-phenol	5000	5000	5000	—	5000	5000	5000
F/8	4-Hydroxy-diphenyl	10 000	10 000	10 000	10 000	10 000	10 000	10 000
F/10	3-Benzyloxy-phenol	(5000)	—	5000	5000	10 000	10 000	10 000
F/11	4-Benzyloxy-phenol	—	—	5000	—	5000	5000	5000
F/12	3-*n*-Hexyloxy-phenol	5000	—	5000	5000	10 000	10 000	10 000
F/13	4-*n*-Hexyloxy-phenol	5000	—	5000	5000	5000	5000	5000
F/14	3-*n*-Octyloxy-phenol	—	—	5000	—	5000	5000	5000
F/15	4-*n*-Octyloxy-phenol	5000	—	5000	5000	5000	5000	5000
F/16	4-Propionyl-phenol	—	—	5000	—	5000	5000	5000

TABELLE 1—*continued.*

Nr.	Verbindungen	*Penicillium simplicissimum*	*Aspergillus niger*	*Trichothecium roseum*	*Candida albicans*	*Achorion quinckeanum*	*Trichophyton gypseum*	*Epidermophyton Kaufman-Wolff*
	II. Die Halogen—Derivate des Phenols							
F 17	2-Chlor-phenol	—	—	5000	—	5000	5000	5000
F 18	4-Chlor-phenol	10 000	5000	10 000	5000	10 000	10 000	10 000
F 19	4-Brom-phenol	10 000	5000	10 000	5000	10 000	10 000	10 000
F 20	2-Jod-4-chlor-phenol	10 000	10 000	10 000	10 000	25 000	25 000	25 000
F 21	2 : 4-Dijod-phenol	10 000	5000	10 000	10 000	10 000	10 000	10 000
F 22	2 : 6-Dibrom-4-chlor-phenol	10 000	10 000	10 000	10 000	10 000	10 000	10 000
F 23	2 : 6-Dijod-4-chlor-phenol	10 000	10 000	10 000	10 000	25 000	25 000	25 000
F 24	2 : 4-Dibrom-6-chlor-phenol	10 000	10 000	10 000	10 000	10 000	25 000	25 000
F 25	2 : 4-Dijod-6-chlor-phenol	10 000	10 000	25 000	10 000	25 000	25 000	25 000
F 26	2 : 4, 6-Tribrom-phenol	10 000	5000	10 000	10 000	10 000	10 000	10 000
F 27	2 : 4, 6-Trijod-phenol	10 000	5000	10 000	5000	10 000	10 000	10 000
	III. Halogen-substituierte Alkyl-, Aryl-, Alkyl-oxy- und Alkoyl-Phenole							
F 28	2-Jod-4-methyl-phenol	10 000	10 000	10 000	5000	10 000	10 000	10 000
F 29	6-Chlor-2 : 4-dimethyl-phenol	10 000	10 000	10 000	10 000	10 000	10 000	10 000
F 30	6-Brom-2 : 4-dimethyl-phenol	10 000	5000	10 000	5000	10 000	10 000	25 000
F 31	6-Jod-2 : 4-dimethyl-phenol	5000	5000	10 000	5000	10 000	10 000	10 000
F 32	2 : 6-Dichlor-4-methyl-phenol	10 000	10 000	10 000	10 000	25 000	25 000	25 000
F 33	2 : 6-Dibrom-4-methyl-phenol	10 000	10 000	10 000	5000	25 000	10 000	25 000
F 34	2 : 6-Dijod-4-methyl-phenol	10 000	10 000	10 000	10 000	25 000	25 000	25 000
F 35	2 : 4-Dijod-5-methyl-phenol	5000	10 000	10 000	5000	25 000	10 000	25 000
F 36	2 : 4 : 6-Tribrom-5-methyl-phenol	10 000	10 000	25 000	10 000	25 000	25 000	25 000
F 37	2 : 4-Dichlor-6-methyl-phenol	10 000	10 000	10 000	10 000	25 000	25 000	25 000
F 38	2 : 4-Dibrom-6-methyl-phenol	10 000	10 000	10 000	5000	25 000	10 000	25 000
F 39	2 : 4-Dijod-6-methyl-phenol	10 000	10 000	10 000	5000	25 000	10 000	25 000
F 40	2 : 6-Dichlor-3 : 4-dimethyl-phenol	10 000	10 000	10 000	10 000	25 000	25 000	25 000
F 41	2 : 6-Dibrom-3 : 4-dimethyl-phenol	5000	5000	25 000	10 000	25 000	25 000	25 000
F 42	2 : 6-Dijod-3 : 4-dimethyl-phenol	5000	5000	25 000	5000	25 000	10 000	25 000
F 43	2-*i*-Propyl-5-methyl-4-brom-phenol	5000	5000	10 000	—	25 000	25 000	25 000
F 44	3-Jod-4-hydroxy-diphenyl	(5000)	—	5000	5000	10 000	5000	5000
F 45	3 : 5-Dichlor-4-hydroxy-diphenyl	—	—	5000	—	5000	5000	5000
F 46	3 : 5-Dibrom-4-hydroxy-diphenyl	5000	—	5000	5000	10 000	10 000	10 000
F 48	2 : 6-Dibrom-3-benzyloxy-phenol	—	—	—	—	10 000	10 000	10 000
F 49	2 : 6-Dibrom-4-benzyloxy-phenol	—	—	—	—	(5000)	(5000)	(5000)
F 50	2 : 6-Dibrom-3-*n*-hexyloxy-phenol	—	—	—	—	5000	5000	5000
F 53	2-Brom-4-propionyl-phenol	5000	10 000	10 000	10 000	25 000	25 000	25 000
F 54	2-Jod-4-propionyl-phenol	—	—	(5000)	—	5000	5000	10 000
	IV. Unsubstituierte und substituierte mehrwertige Phenole							
F 55	Pyrocatechin	—	—	—	—	10 000	10 000	10 000
F 57	Hydrochinon	—	—	—	—	10 000	10 000	10 000
F 58	Pyrogallol	—	—	—	—	25 000	25 000	25 000
F 62	4-*n*-Hexyl-resorzin	10 000	5000	10 000	—	10 000	10 000	10 000
F 64	Tetrabrom-pyrocatechin	—	—	—	—	5000	5000	5000
F 65	2 - 6-Dibrom-resorzin	5000	(5000)	10 000	—	25 000	25 000	25 000
F 67	2 : 6-Dibrom-5-methyl-resorzin	5000	—	5000	—	5000	5000	10 000
F 68	2 : 6-Dibrom-4-*n*-hexyl-resorzin	—	—	5000	—	5000	10 000	10 000
	V. Phenol-aldehyde							
F 71	2-Hydroxy-benzaldehyd	5000	5000	10 000	5000	25 000	10 000	25 000
F 72	4-hydroxy-benzaldehyd	—	—	—	—	5000	5000	5000
F 74	2-Hydroxy-5-brom-benzaldehyd	5000	5000	10 000	—	10 000	10 000	10 000
	VI. Phenol-carbonsäuren und ihre Ester							
F 79	2-Hydroxy-5-jod-benzoesäure	—	—	—	—	5000	5000	5000

TABELLE 1—*continued.*

Nr.	Verbindungen	*Penicillium simplicissimum*	*Aspergillus niger*	*Trichotheclium roseum*	*Candida albicans*	*Achorion quinckeanum*	*Trichophyton gypseum*	*Epidermophyton Kaufman-Wolff*
F 80	2-Hydroxy-3 : 5-dijod-benzoesäure	—	—	5000	—	5000	5000	5000
F 83	3 : 5-Dijod-4-hydroxy-benzoesäure	—	—	—	—	5000	5000	5000
F 87	2-Hydroxy-benzoesäure-*n*-butyl-ester	—	—	(5000)	—	10 000	10,000	10 000
F 88	2-Hydroxy-benzoesäure-*n*-amyl-ester	—	—	—	—	10 000	10 000	10 000
F 89	2-Hydroxy-benzoesäure-*n*-hexyl-ester	—	—	—	—	10 000	10 000	10 000
F 90	2-Hydroxy-benzoesäure-phenyl-ester	—	—	5000	—	10 000	10 000	10 000
F 91	4-Hydroxy-benzoesäure-methyl-ester	—	—	—	—	5000	5000	5000
F 92	4-Hydroxy-benzoesäure-*n*-propyl-ester	5000	—	5000	—	10 000	10 000	10 000
F 93	4-Hydroxy-benzoesäure-*n*-butyl-ester	5000	—	5000	—	10 000	10 000	25 000
F 94	4-Hydroxy-benzoesäure-*n*-amyl-ester	—	—	(5000)	—	10 000	10 000	25 000
F 95	4-Hydroxy-benzoesäure-*n*-hexyl-ester	—	—	(5000)	—	25 000	10 000	25 000
F 96	2-Hydroxy-5-brom-benzoesäure-methyl-ester	(5000)	5000	5000	—	5000	10 000	10 000
F 97	2-Hydroxy-5-brom-benzoesäure-*n*-butyl-ester	—	—	—	—	5000	5000	10 000
F 98	2-Hydroxy-5-brom-benzoesäure-*n*-amyl-ester	—	—	—	—	5000	5000	5000
F 99	2-Hydroxy-5-brom-benzoesäure-*n*-hexyl-ester	—	—	—	—	(5000)	(5000)	(5000)
F 101	2-Hydroxy-5-jod-benzoesäure-methyl-ester	—	—	10 000	5000	10 000	10 000	10 000
F 102	3-Brom-4-hydroxy-benzoesäure-methyl-ester	—	—	5000	—	10 000	10 000	10 000
F 103	3-Brom-4-hydroxy-benzoesäure-*n*-propyl-ester	—	—	5000	—	25 000	25 000	25 000
F 104	3-Brom-4-hydroxy-benzoesäure-*n*-butyl-ester	—	—	5000	—	25 000	10 000	25 000
F 105	3-Brom-4-hydroxy-benzoesäure-*n*-amyl-ester	—	—	—	—	10 000	10 000	10 000
F 106	3-Brom-4-hydroxy-benzoesäure-*n*-hexyl-ester	—	—	—	—	10 000	10 000	10 000
F 107	3-Jod-4-hydroxy-benzoesäure-*n*-propyl-ester	—	—	5000	—	10 000	10 000	10 000
	VII. Naphtole und ihre Derivate							
F 109	1-Naphtol	10 000	10 000	10 000	10 000	10 000	10 000	10 000
F 110	2-Naphtol	10 000	10 000	10 000	5000	10 000	10 000	10 000
F 111	2-Hydroxy-1-naphtaldehyd	10 000	10 000	10 000	10 000	10 000	10 000	10 000
F 112	2-Chlor-1-naphtol	10 000	10 000	10 000	10 000	10 000	10 000	10 000
F 113	2-Brom-1-naphtol	10 000	5000	10 000	5000	25 000	25 000	25 000
F 114	1-Chlor-2-napthol	10 000	10 000	10 000	10 000	25 000	10 000	25 000
F 115	1-Brom-2-naphtol	10 000	10 000	10 000	10 000	25 000	25 000	25 000
F 119	2 : 2'-Dihydroxy-1 : 1'-dinaphtyl-methan	—	—	—	—	5000	5000	5000
	VIII. Basischen Substituent enthaltende Phenole und Naphtole							
F 120	2-Amino-phenol	10 000	10 000	10 000	10 000	25 000	25 000	25 000
F 121	4-Amino-phenol	—	—	—	—	25 000	25 000	25 000
F 122	4-Methylamino-phenol	—	—	—	—	25 000	25 000	25 000
F 124	2 : 4 : 6-Tribrom-3-diaethylamino-phenol	—	—	5000	—	5000	5000	5000
F 125	2 : 4-Diamino-phenol dihydrochlorid	—	—	—	—	5000	5000	5000
F 133	1-Diaethylamino-methyl-2-naphtol	—	—	—	—	5000	5000	5000
F 134	1-Di-(*n*-butyl)-amino-methyl-2-naphtol	—	—	—	—	5000	5000	5000
F 135	1-Piperidino-methyl-2-naphtol	—	—	—	—	5000	5000	5000
F 136	1-(2'-Hydroxy-benzyl)-2-(2'-hydroxy-phenyl)-imidazolin-(4 : 5)	5000	—	5000	10 000	10 000	10 000	10 000
F 137	1-(2'-Hydroxy-5'-brom-benzyl)-2-(2'-hydroxy-5-brom-phenyl)-imidazolin (4 : 5)	5000	—	5000	5000	10 000	5000	10 000
F 139	1-(2'-Hydroxy-benzyl)-2-(2'-hydroxy-phenyl-) benzimidazol	(5000)	—	5000	—	10 000	10 000	10 000

TABELLE 1—*continued.*

Nr.	Verbindungen	*Penicillium simplicissimum*	*Aspergillus niger*	*Trichothecium roseum*	*Candida albicans*	*Achorion quinckeanum*	*Trichophyton gypseum*	*Epidermophyton Kaufman-Wolff*
	IX. Derivate von Hydroxyazobenzolen							
F/145	4-Hydroxy-azobenzol	(5000)	—	5000	—	5000	5000	5000
F/146	4-Hydroxy-3-methyl-azobenzol	—	—	(5000)	—	(5000)	(5000)	(5000)
F/149	4-Hydroxy-3-chlor-azobenzol	(5000)	—	10 000	5000	10 000	10 000	10 000
F/150	4-Hydroxy-3-methyl-4′-clor azobenzol	—	—	5000	—	5000	5000	5000
F/152	4-Hydroxy-3 : 4′-dichlor-azobenzol	—	—	5000	5000	10 000	10 000	10 000
	X. Durch Schwefel-oder Methylen-Brücken vereinigte Di- und Polyphenole							
F/178	2 : 2′-Dihydroxy-5 : 5′-dichlor-diphenyl-methan	—	—	—	—	5000	5000	5000
F/179	Poly-(2-hydroxy-5-chlor-phenyl-disulfid)	(5000)	—	5000	—	10 000	10 000	10 000
F/183	Poly-(2-hydroxy-5-chlor-phenyl-methan)	—	—	—	—	(5000)	(5000)	(5000)
	XI. Phenolische Hydroxyl-Gruppe enthaltende sonstige Verbindungen							
F/186	1 : 2 : 9-Trihydroxy-anthrazen	—	—	—	(5000)	5000	5000	5000
F/189	Stilböstrol	—	—	—	—	5000	5000	5000
F/202	Apomorphin hydrochlorid	—	—	—	—	5000	5000	5000
	XII. Acetate von Phenol-Derivaten							
F/207	1-Acetyloxy-4-chlor-benzol	10 000	—	10 000	(5000)	10000	10 000	10 000
F/208	1-Acetyloxy-2-*i*-propyl-5-methyl-benzol	5000	—	5000	—	5000	5000	5000
F/209	4-Acetyloxy-benzoesäure-*n*-propyl-ester	5000	—	5000	—	10 000	10 000	10 000
F/210	4-Acetyloxy-diphenyl	10 000	5000	10 000	5000	10 000	10 000	10 000
F/211	1-Acetyloxy-naphtalin	10 000	5000	10 000	(5000)	10 000	10 000	10 000
F/212	2-Acetyloxy-naphtalin	10 000	5000	10 000	—	10 000	10 000	10 000
F/213	1-Chlor-2-acetyloxy-naphtalin	10 000	10 000	10 000	5000	10 000	10 000	1 0000
	XIII. Alkyl-äther von substituierten Phenolen							
F/214	1-Benzyloxy-2-methyl-benzol	—	—	5000	—	5000	5000	5000
F/217	1-Benzyloxy-4-methyl-benzol	—	—	5000	—	5000	5000	5000
F/219	1-Allyloxy-2-*i*-propyl-5-methyl-benzol	—	—	—	—	5000	5000	10 000
F/222	1-Benzyloxy-2-*i*-propyl-5-methyl-benzol	—	—	—	—	5000	5000	5000
F/225	1-Allyloxy-4-chlor-benzol	—	—	10 000	—	10 000	10 000	10 000
F/227	1-Aethoxy-4-jod-benzol	—	—	(5000)	—	5000	5000	5000
F/230	1-Benzyloxy-2 : 4-dibrom-6-methyl-benzol	—	—	(5000)	—	5000	5000	5000
F/231	1-Aethoxy-2 : 4-dijod-benzol	—	—	5000	—	5000	5000	5000
F/232	1-Benzyloxy-2-jod-4-chlor-benzol	—	—	—	—	5000	(5000)	5000
F/237	1-Benzyloxy-2 : 6-dijod-4-chlor-benzol	—	—	(5000)	—	5000	5000	5000
F/239	1-Aethoxy-2 : 4, 6-trijod-benzol	—	—	5000	—	5000	5000	5000
F/240	4-Aethoxy-diphenyl	—	—	10 000	—	10 000	10 000	10 000
F/244	2 : 4-Diaethoxy-1-*n*-hexyl-benzol	—	—	—	—	5000	5000	5000
F/245	2 : 4-Di-(β-hydroxy-aethoxy)-1-*n*-hexyl-benzol	—	—	—	—	5000	5000	5000
	XIV. Alkyl-äther von Naphtolen und ihren halogensubstituierten Derivaten							
F/246	1-Aethoxy-naphtalin	5000	(5000)	10 000	—	10 000	10 000	10 000
F/247	1-Allyloxy-naphtalin	—	—	10 000	—	10 000	10 000	10 000
F/248	1-*n*-Hexyloxy-naphtalin	—	—	5000	—	5000	5000	5000
F/252	2-Aethoxy-naphtalin	5000	—	10 000	—	10 000	10 000	10 000
F/253	2-(β-Hydroxy-aethoxy)-naphtalin	(5000)	—	(5000)	—	5000	5000	5000
F/254	2-Allyloxy-naphtalin	—	—	10 000	—	10 000	10 000	10 000
F/255	2-*n*-Hexyloxy-naphtalin	—	—	5000	—	5000	5000	(5000)
F/259	1-Aethoxy-2-brom-naphtalin	—	—	10 000	—	10 000	10 000	10 000
F/260	2-Aethoxy-1-brom-naphtalin	—	—	5000	—	10 000	10 000	10 000

TABELLE 2. PHENOL-DERIVATE, WELCHE IN FLÜSSIGEM SERUM-MAISCHE NÄHRBODEN AUF JEDE UNTERSUCHTE PILZSTÄMME VOLLSTÄNDIG UNWIRKSAM SIND

F/1	Phenol
F/9	2-Methoxy-phenol
F/47	3:5-Dijod-4-hydroxy-diphenyl
F/51	2:6-Dibrom-3-*n*-octyloxy-phenol
F/52	2:6-Dibrom-4-*n*-octyloxy-phenol
F/56	Resorzin
F/59	Phlorogluzin
F/60	5-Methyl-resorzin
F/61	5:5-Dimethyl-4:5-dihydro-resorzin
F/63	Phloridzin
F/66	2:6-Dijod-resorzin
F/69	2:6-Dijod-4-*n*-hexyl-resorzin
F/70	Tribrom-phlorogluzin
F/73	3-Methoxy-4-hydroxy-benzaldehyd
F/75	2-Hydroxy-benzoesäure
F/76	4-Hydroxy-benzoesäure
F/77	3:4:5-Trihydroxy-benzoesäure
F/78	2-Hydroxy-5-brom-benzoesäure
F/81	3-Brom-4-hydroxy-benzoesäure
F/82	3-Jod-4-hydroxy-benzoesäure
F/84	2-Hydroxy-4-amino-benzoesäure
F/85	2-Hydroxy-4-dichloracetylamino-benzoesäure
F/86	2-Hydroxy-benzoesäure-methyl-ester
F/100	2-Hydroxy-5-brom-benzoesäure-phenyl-ester
F/108	3-Hydroxy-4-amino-benzoesäure-methyl-ester
F/116	1:3-Dihydroxy-naphtalin
F/117	1:1′-Dihydroxy-2:2′-dinaphtyl
F/118	2:2′-Dihydroxy-1:1′-dinaphtyl
F/123	3-Diaethylamino-phenol
F/126	1-(4′-Hydroxy-phenyl)-1-hydroxy-2-methylamino-aethan hydrochlorid
F/127	1-(3′-Hydroxy-phenyl)-2-amino-propan hydrochlorid
F/128	1-(3′:4′-Dihydroxy-phenyl)-1-hydroxy-2-*i*-propylamino-aethan hydrochlorid
F/129	6-Diaethylamino-methyl-2:4-dimethyl-phenol
F/130	6-Di-(*n*-butyl)-amino-methyl-2:4-dimethyl-phenol
F/131	6-Piperidino-methyl-2:4-dimethyl-phenol
F/132	2:5-Di-(diaethylamino-methyl)-hydrochinon
F/138	1-(4′-Hydroxy-benzyl)-2-(4′-hydroxy-phenyl)-imidazolin-(4:5)
F/140	1-(2′-Hydroxy-5′-brom-benzyl)-2-(2′-hydroxy-5′-brom-phenyl)-benzimid-azol
F/141	1-(4′-Hydroxy-benzyl)-2-(4′-hydroxy-phenyl)-benzimidazol
F/142	Salicyl-aldehyd-aminoguanidon nitrat
F/143	5-Brom-salicyl-aldehyd-amino-guanidon nitrat
F/144	4-Hydroxy-benzaldehyd-amino-guanidon nitrat
F/147	4-Hydroxy-2-methyl-azobenzol
F/148	4-Hydroxy-2-methyl-5-*i*-propyl-azobenzol
F/151	4-Hydroxy-2-methyl-4′-chlorazobenzol
F/153	4-Hydroxy-azobenzol-3-carbonsäure
F/154	4-Hydroxy-4′-nitro-azobenzol-3-carbonsäure
F/155	4-Hydroxy-3′-nitro-azobenzol-3-carbonsäure
F/156	2-Hydroxy-5-methyl-azobenzol
F/157	2-Hydroxy-5-chlor-azobenzol
F/158	2-Hydroxy-5-methyl-4-chlor-azobenzol
F/159	2-Hydroxy-5:4′-dichlor-azobenzol
F/160	2:4-Dihydroxy-azobenzol
F/161	2:4-Dihydroxy-5-*n*-hexyl-4′-chlor-azo-benzol
F/162	Chrysamin
F/163	4-Phenylazo-1-naphtol
F/164	1-Phenylazo-2-naphtol
F/165	2-Chlor-4-(4′-chlor-phenyl-azo)-1-naphtol
F/166	4-Chlor-phenol-2-sulfonsäure-Na
F/167	Sulfosalicyl-säure
F/168	4-Hydroxy-diphenyl-3-sulfonsäure-Na
F/169	1-Naphtol-4-sulfonsäure-Na
F/170	2-Naphtol-6-sulfonsäure-Na
F/171	1-Amino-2-naphtol-4-sulfonsäure
F/172	1-Phenylazo-2-naphtol-6-sulfonsäure-Na
F/173	2-Hydroxy-5-methyl-azobenzol-4′-sulfonsäure
F/174	Brillant-gelb
F/175	2:2′-Dihydroxy-diphenyl-disulfid
F/176	2:2′-Dihydroxy-5:5′-dimethyl-diphenyl-disulfid
F/177	2:2′-Dihydroxy-5,5′-dichlor-diphenyl-disulfid
F/180	Poly-(2-hydroxy-phenyl-methan)
F/181	Poly-(2-hydroxy-3-methyl-phenyl-methan)
F/182	Poly-(2-hydroxy-5-methyl-phenyl-methan)
F/184	9-Anthron
F/185	10-Brom-9-anthron
F/187	2:2′-Dihydroxy-3:3′-di-*i*-propyl-5:5′-dijod-6:6′-dimethyl-diphenyl
F/188	D:L-Thyroxin
F/190	α-Phenyl-β-(3:5-dijod-4-hydroxy-phenyl)-propionsäure
F/191	Rosol-säure
F/192	Rubrophen
F/193	Phenolphtalein
F/194	Thymolphtalein
F/195	Brom-thymol-blau
F/196	Tetrabrom-phenolphtalein
F/197	Tetrajod-phenolphtalein
F/198	Kresol-rot
F/199	Eriochromcyanin R
F/200	Fluorescein
F/201	Eozin
F/203	Haematoxylin
F/204	7-Chlor-tetracyclin hydrochlorid
F/205	5-Oxy-tetracyclin hydrochlorid
F/206	7-(4′-Chlor-phenyl-azo)-5-oxy-tetracyclin
F/215	1-Aethoxy-4-methyl-benzol
F/216	1-Allyloxy-4-methyl-benzol
F/218	1-Aethoxy-2-*i*-propyl-5-methyl-benzol
F/220	1-*n*-Hexyloxy-2-*i*-propyl-5-methyl-benzol
F/221	1-*n*-Octyloxy-2-*i*-propyl-5-methyl-benzol

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TABELLE 2—*continued.*

F/223	1-Aethoxy-4-chlor-benzol	F/238	1-Aethoxy-2:4:6-tribrom-benzol
F/224	1-(β-Hydroxy-aethoxy)-4-chlor-benzol	F/241	4-Benzyloxy-diphenyl
F/226	1-Benzyloxy-4-chlor-benzol	F/242	4-Aethoxy-3:5-dibrom-diphenyl
F/228	1-Benzyloxy-2-jod-4-methyl-benzol	F/243	1:2-Dimethoxy-benzol
F/229	1-Aethoxy-2-*i*-propyl-5-methyl-4-brom-benzol	F/249	1-*n*-Octyloxy-naphtalin
		F/250	1-*n*-Dodecyloxy-naphtalin
F/233	1-Aethoxy-2:6-dijod-4-methyl-benzol	F/251	1-Benzyloxy-naphtalin
F/234	1-Allyloxy-2:6-dijod-4-methyl-benzol	F/256	2-*n*-Octyloxy-naphtalin
F/235	1-Benzyloxy-2:6-dijod-4-methyl-benzol	F/257	2-*n*-Dodecyloxy-naphtalin
F/236	1-Benzyloxy-2:4-dijod-6-methyl-benzol	F/258	2-Benzyloxy-naphtalin

TABELLE 3. DIE FUNGISTATISCHE WIRKUNG VON PHENOL-DERIVATEN AUF FESTEM MAISCHE-AGAR NÄHRBODEN

Nr.	Verbindungen	*Penicillium simplicissimum*	*Aspergillus niger*	*Trichothecium roseum*	*Candida albicans*	*Saccharomyces cerevisiae*
F/8	4-Hydroxy-diphenyl	15	20	17	10	10
F/12	3-*n*-Hexyloxy-phenol	16	10	15	13	12
F/13	4-*n*-Hexyloxy-phenol	17	17	12	16	12
F/20	2-Jod-4-chlor-phenol	23	25	27	23	23
F/21	2:4-Dijod-phenol	25	25	30	25	22
F/22	2:6-Dibrom-4-chlor-phenol	25	27	30	20	22
F/23	2:6-Dijod-4-chlor-phenol	23	37	37	25	23
F/24	2:4-Dibrom-6-chlor-phenol	30	36	32	32	27
F/25	2:4-Dijod-6-chlor-phenol	30	36	32	35	27
F/26	2:4:6-Tribrom-phenol	35	37	53	30	32
F/27	2:4:6-Trijod-phenol	17	21	20	25	17
F/30	6-Brom-2:4-dimethyl-phenol	20	32	32	20	18
F/31	6-Jod-2:4-dimethyl-phenol	12	13	15	12	10
F/32	2:6-Dichlor-4-methyl-phenol	—	—	22	22	10
F/33	2:6-Dibrom-4-methyl-phenol	17	10	25	10	15
F/34	2:6-Dijod-4-methyl-phenol	20	25	23	20	18
F/35	2:4-Dijod-5-methyl-phenol	16	17	16	16	15
F/36	2:4:6-Tribrom-5-methyl-phenol	16	15	20	12	12
F/37	2:4-Dichlor-6-methyl-phenol	12	—	18	12	10
F/38	2:4-Dibrom-6-methyl-phenol	32	37	37	17	17
F/39	2.4-Dijod-6-methyl-phenol	20	35	20	20	20
F/40	2:6-Dichlor-3:4-dimethyl-phenol	15	—	12	15	10
F/41	2:6-Dibrom-3:4-dimethyl-phenol	15	12	20	26	15
F/42	2:6-Dijod-3:4-dimethyl-phenol	10	22	17	15	13
F/43	2-*i*-Propyl-5-methyl-4-brom-phenol	10	10	12	12	12
F/44	3-Jod-4-hydroxy-diphenyl	13	15	17	10	14
F/45	3:5-Dichlor-4-hydroxy-diphenyl	10	10	22	10	12
F/46	3:5-Dibrom-4-hydroxy-diphenyl	10	12	13	10	—
F/53	2-Brom-4-propionyl-phenol	24	17	25	20	28
F/54	2-Jod-4-propionyl-phenol	20	10	17	10	10
F/62	4-*n*-Hexyl-resorzin	28	18	30	12	12
F/64	Tetrabrom-pyrocatechin	18	18	15	17	18
F/65	2:6-Dibrom-resorzin	10	10	23	15	11
F/67	2:6-Dibrom-5-methyl-resorzin	15	20	17	15	17
F/93	4-Hydroxy-benzoesäure-*n*-butyl-ester	11	15	12	17	12

TABELLE 3—*continued.*

Nr.	Verbindungen	*Penicillium simplicissimum*	*Aspergillus niger*	*Trichothecium roseum*	*Candida albicans*	*Saccharomyces cerevisiae*
F/102	3-Brom-4-hydroxy-benzoesäure-methyl-ester	15	25	15	17	10
F/103	3-Brom-4-hydroxy-benzoesäure-*n*-propyl-ester	15	23	15	17	10
F/104	3-Brom-4-hydroxy-benzoesäure-*n*-butyl-ester	12	12	12	14	—
F/109	1-Naphtol	20	15	20	—	—
F/110	2-Naphtol	20	15	22	—	—
F/111	2-Hydroxy-1-naphtaldehyd	15	15	18	—	—
F/112	2-Chlor-1-naphtol	34	33	33	25	12
F/113	2-Brom-1-naphtol	10	10	15	10	10
F/114	1-Chlor-2-naphtol	46	40	40	25	12
F/115	1-Brom-2-naphtol	20	20	33	20	25
F/120	2-Amino-phenol	30	—	20	—	13
F/149	4-Hydroxy-3-chlor-azobenzol	15	15	12	20	15
F/150	4-Hydroxy-3-methyl-4′-chlor-azobenzol	12	—	22	12	12
F/151	4-Hydroxy-2-methyl-4′-chlor-azobenzol	10	—	17	10	12
F/152	4-Hydroxy-3:4′-dichlor-azobenzol	12	—	20	15	12
F/212	2-Acetyloxy-naphtalin	17	15	—	—	10
F/213	1-Chlor-2-acetyloxy-naphtalin	20	17	—	10	10

Untersuchungen über den Wirkungsmechanismus der Phenol-Derivate

Schraufstätter und seine Mitarbeiter haben nachgewiesen, dass die fungistatische Wirkung des 2:2′-Dioxy-5:5′-dichlor-diphenyl-sulfids durch die Zugabe des an 55 °C Temperatur inaktivierten menschlichen Serums zum Nährboden bedeutend geschwächt werden kann.[31] Durch Serum wird—laut der Fachliteratur—auch die fungistatische Wirkung des N-*n*-Butyl-3-phenyl-salicylamids[31] und des *p*-Hydroxy-benzoesäure-*n*-propyl-ester[37] erniedrigt.

Trotzdem wurde aber die Beeinflussung der fungistatischen Wirkung der Phenole durch Serum, sowie die Abhängigkeit dieses Einflusses von dem chemischen Aufbau auf breiteren Grundlagen noch nicht untersucht. Deshalb haben wir im Laufe unserer Forschungen mit Rücksicht auf die fungistatische Wirkung der auf serumhaltigem flüssigem Nährboden sich schon wirksam erwiesenen Phenol-Derivate *auch auf serumfreiem flüssigem Maische-Nährboden*, ausserdem aber auch *auf festem Maische-Agar Nährboden mit* 10% *Rinderserum-Gehalt* Untersuchungen vorgenommen. Wir suchten zu erforschen, ob es zwischen der auf serumfreiem und serumhaltigem Nährboden ausgeübten fungistatischen Wirkung der Phenol-Derivate sowohl auf flüssigem, als auch auf festem Nährboden-einen bedeutenden Unterschied geben kann. Wir haben unsere Forschungen auch in der Richtung ausgedehnt, inwieweit andere Zellbestandteile ausser den Eiweissen, wie z.B. das Lecithin, Lanolin (=Cholesterin-fettsäuren-ester), Cholesterin und einfache Fette (z.B. Sonnenblumenöl) die fungistatische Wirkung der bei unseren Experimenten wirksam erwiesenen Phenole beeinflussen. Ausserdem haben wir Untersuchungen bezüglich dessen unternommen, inwieweit die fungistatische Wirkung der verschiedenen Phenole (auf serumfreiem Nährboden) von dem pH des Kulturmediums abhängig ist.

Alle diese Untersuchungen wurden deshalb durchgeführt, um dadurch zur Erkenntnis des biochemischen Wirkungsmechanismus der Phenol-Derivate mit fungistatischer Wirkung näher zu kommen und zugleich die Zusammenhänge zwischen der Intensität der fungistatischen Wirkung und der chemischen Struktur innerhalb dieser Gruppe von Verbindungen aufklären zu können.

Die angewandte Methode war identisch mit der vorher geschriebenen. Aber zu diesen Versuchen anwendeten wir nicht nur serumhaltigen flüssigen Maische-Nährboden und festen Maische-Agar sondern auch serumlosen Maische-Nährboden und 1% Rinderserum enthaltenden festen Maische-Agar; ausserdem wurden Maische Nährboden angewandt, welche enthielten noch (als Zugaben) 0,25% Lecithin, Lanolin, Cholesterin, bzw. Sonnenblumenöl.

Zu unseren auf flüssigen Nährbodenarten unternommenen Untersuchungen haben wir als Test-Organismus *Penicillium simplicissimum*, auf den festen Maische-Agar-Nährbodenarten durchgeführten Versuchen ausserdem noch *Trichothecium roseum* und *Saccharomyces cerevisiae* verwendet.

Die Ergebnisse, die wir über den biochemischen Wirkungsmechanismus der Phenol-Derivate erzielt haben, sind in den Tabellen 4 und 5 zusammengefasst.

Wir untersuchten auf festem Maische-Agar-Nährboden die durch Serum verursachte Beeinflussbarkeit der fungistatischen Wirkung jeder Phenol-Derivate welche in der Tabelle 3 aufgeführt sind. Unsere Versuche gaben das Ergebnis, dass die auf

TABELLE 4. DIE BEEINFLUSSUNG DER AUF DAS *Penicillium simplicissimum* AUSGEÜBTEN FUNGISTATISCHEN WIRKUNG DER PHENOL-DERIVATE DURCH DIE ZUGABE VON VERSCHIEDENEN SUBSTANZEN ZUM FLÜSSIGEN MAISCHE-NAHRBODEN.

Nr.	Verbindungen	—	10% Rinder serum	0,25% Lecit-hin	0,25% Lano-lin	0,25% Chole-sterin	0,25% Sonnen-blumenöl
F/7	2-*i*-Propyl-5-methyl-phenol	10 000	5000	5000	—	10 000	—
F/8	4-Hydroxy-diphenyl	25 000	10 000	10 000	10 000	25 000	10 000
F/12	3-*n*-Hexyloxy-phenol	10 000	5000	5000	—	10 000	—
F/13	4-*n*-Hexyloxy-phenol	10 000	5000	5000	—	10 000	—
F/18	4-Chlor-phenol	10 000	10 000	5000	10 000	10 000	10 000
F/19	4-Brom-phenol	10 000	10 000	5000	10 000	10 000	10 000
F/20	2-Jod-4-chlor-phenol	25 000	10 000	10 000	10 000	25 000	10 000
F/21	2:4-Dijod-phenol	25 000	10 000	10 000	(5000)	25 000	—
F/22	2:6-Dibrom-4-chlor-phenol	50 000	10 000	10 000	10 000	50 000	10 000
F/23	2:6-Dijod-4-chlor-phenol	50 000	10 000	10 000	10 000	50 000	10 000
F/24	2:4-Dibrom-6-chlor-phenol	25 000	10 000	10 000	10 000	25 000	10 000
F/25	2:4-Dijod-6-chlor-phenol	25 000	10 000	10 000	5000	25 000	10 000
F/26	2:4:6-Tribrom-phenol	50 000	10 000	10 000	(5000)	50 000	(5000)
F/27	2:4:6-Trijod-phenol	25 000	10 000	10 000	5000	25 000	—
F/28	2-Jod-4-methyl-phenol	10 000	10 000	5000	5000	10 000	5000
F/29	6-Chlor-2:4-dimethyl-phenol	10 000	10 000	10 000	5000	10 000	5000
F/30	6-Brom-2:4-dimethyl-phenol	25 000	10 000	5000	5000	25 000	5000
F/31	6-Jod-2:4-dimethyl-phenol	25 000	5000	5000	—	25 000	—
F/32	2:6-Dichlor-4-methyl-phenol	25 000	10 000	10 000	5000	25 000	10 000
F/33	2:6-Dibrom-4-methyl-phenol	25 000	10 000	10 000	5000	25 000	5000
F/34	2:6-Dijod-4-methyl-phenol	25 000	10 000	5000	—	25 000	—
F/35	2:4-Dijod-5-methyl-phenol	25 000	5000	10 000	—	25 000	—
F/36	2:4:6-Tribrom-5-methyl-phenol	50 000	10 000	10 000	—	50 000	—
F/37	2:4-Dichlor-6-methyl-phenol	25 000	10 000	10 000	5000	25 000	10 000
F/38	2,4-Dibrom-6-methyl-phenol	25 000	10 000	10 000	5000	25 000	5000
F/39	2:4-Dijod-6-methyl-phenol	25 000	10 000	5000	5000	25 000	—
F/40	2:6-Dichlor-3:4-dimethyl-phenol	10 000	10 000	10 000	5000	10 000	—

TABELLE 4—*continued.*

Nr.	Verbindungen	—	10% Rinder-serum	0,25% Lecit-hin	0,25% Lano-lin	0,25% Chole-sterin	0,25% Sonnen-blumenol
F/41	2:6-Dibrom-3:4-dimethyl-phenol						
F/42	2:6-Dijod-3:4-dimethyl-phenol	25 000	5000	5000	—	25 000	—
F/43	2-*i*-Propyl-4-brom-5-methyl-phenol	10 000	5000	(5000)	—	10 000	—
		25 000	5000	5000	—	26 000	(5000)
F/53	2-Brom-4-propionyl-phenol	10 000	5000	10 000	10 000	10 000	10 000
F/62	4-*n*-Hexyl-resorzin	25 000	10 000	10 000	10 000	25 000	10 000
F/64	Tetrabrom-pyrokatechin	25 000	—	—	—	25 000	10 000
F/65	2:6-Dibrom-resorzin	10 000	5000	5000	(5000)	10 000	5000
F/67	2:6-Dibrom-5-methyl-resorzin	10 000	5000	5000	5000	10 000	5000
F/68	2:6-Dibrom-4-*n*-hexyl-resorzin	10 000	—	—	—	10 000	—
F/74	2-Hydroxy-5-brom-benzal-dehyd	25 000	5000	5000	5000	10 000	5000
F/92	4-Hydroxy-benzoesäure-*n*-propyl-ester	10 000	5000	(5000)	(5000)	10 000	5000
F/93	4-Hydroxy-benzoesäure-*n*-butyl-ester	10 000	5000	(5000)	(5000)	10 000	(5000)
F/102	3-Brom-4-hydroxy-benzoe-säure-methyl-ester	10 000	—	—	—	10 000	—
F/103	3-Brom-4-hydroxy-benzoe-säure-*n*-propyl-ester	10 000	—	—	—	10 000	—
F/109	1-Naphtol	25 000	10 000	10 000	5000	25 000	5000
F/110	2-Naphtol	25 000	10 000	10 000	10 000	25 000	10 000
F/112	2-Chlor-1-naphtol	25 000	10 000	5000	5000	25 000	5000
F/113	2-Brom-1-naphtol	25 000	10 000	5000	5000	25 000	5000
F/114	1-Chlor-2-naphtol	25 000	10 000	10 000	5000	25 000	5000
F/115	1-Brom-2-naphtol	25 000	10 000	5000	5000	25 000	5000
F/149	4-Hydroxy-3-chlor-azobenzol	25 000	(5000)	(5000)	—	25 000	—
F/207	1-Acetyloxy-4-chlor-benzol	10 000	10 000	10 000	5000	10 000	5000
F/210	4-Acetyloxy-diphenyl	25 000	10 000	5000	—	25 000	—
F/211	1-Acetyloxy-naphtalin	10 000	10 000	10 000	5000	10 000	5000
F/212	2-Acetyloxy-naphtalin	10 000	10 000	10 000	5000	10 000	5000
F/213	1-Chlor-2-acetyloxy-naphtalin	25 000	10 000	10 000	5000	25 000	5000
F/246	1-Aethoxy-naphtalin	10 000	5000	5000	—	5000	—
F/252	2-Aethoxy-naphtalin	10 000	5000	—	—	5000	—

TABELLE 5. DER EINFLUSS DES pH DES NÄHRBODENS AUF DIE FUNGISTATISCHE WIRKUNG DER PHENOL-DERIVATE GEGEN DAS *Penicillium simplicissimum*

Nr.	Verbindungen	pH = 4,0	pH = 6,0	pH = 8,0
F/8	4-Hydroxy-diphenyl	25 000	25 000	25 000
F/18	4-Chlor-phenol	10 000	10 000	10 000
F/20	2-Jod-4-chlor-phenol	50 000	25 000	25 000
F/22	2:6-Dibrom-4-chlor-phenol	100 000	50 000	5000
F/23	2:6-Dijod-4-chlor-phenol	50 000	50 000	10 000
F/32	2:6-Dichlor-4-methyl-phenol	25 000	25 000	25 000
F/40	2:6-Dichlor-3:4-dimethyl-phenol	10 000	10 000	10 000
F/41	2:6-Dibrom-3:4-dimethyl-phenol	25 000	25 000	10 000
F/42	2:6-Dijod-3:4-dimethyl-phenol	10 000	10 000	10 000
F/53	2-Brom-4-propionyl-phenol	25 000	10 000	10 000
F/62	4-*n*-Hexyl-resorzin	25 000	25 000	25 000
F/64	Tetrabrom-pyrokatechin	25 000	25 000	(5000)
F/71	2-Hydroxy-benzaldehyd	5000	5000	5000
F/93	4-Hydroxy-benzoesäure-*n*-butyl-ester	10 000	10 000	10 000
F/104	3-Brom-4-hydroxy-benzoesäure-*n*-butyl-ester	10 000	10 000	10 000
F/109	1-Naphtol	25 000	25 000	25 000
F/110	2-Naphtol	25 000	25 000	25 000
F/114	1-Chlor-2-naphtol	25 000	25 000	25 000
F/115	1-Brom-2-naphtol	25 000	25 000	25 000

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das *Penicillium simplicissimum*, *Trichothecium roseum* und *Saccharomyces cerevisiae* ausgeübte fungistatische Wirkung jeder Verbindungen der Tabelle 3 (ausgenommen die des *o*-Amino-phenols, F/120)–auf festem Maische-Agar-Nährboden durch Serum gleichmässig ganz aufgehoben wurde.

BESPRECHUNG DER ERGEBNISSE

Bei der Übersicht der auf flüssigem Serum-Maische-Nährboden ausgeführten Untersuchungen fällt es gleich in die Augen, dass zahlreiche Phenol-Derivate selbst in einer 2–2,5-mal, einige sogar in mehr als fünfmal grösserer Verdünnung wirksam sind gegen das *Trichothecium roseum*, *Achorion quinckeanum*, *Trichophyton gypseum* und *Epidermophyton Kaufman-Wolff*, wie auf das *Penicillium simplicissimum* und *Aspergillus niger*, oder auf das *Candida albicans*. Diese Verschiedenheit der Sensibilität der zwei Gruppen von den angeführten Pilzstämme besteht freilich nicht gegenüber einem jedem Phenol-Derivat mit fungistatischer Wirkung; sie lässt sich also für keine Gesetzmässigkeit von allgemeiner Geltung halten, die keine Ausnahme erlaubt, sondern kann vielmehr für eine sehr häufige (und eben deshalb sehr charakteristische) Erscheinung genommen werden, woraus man den Schluss ziehen kann, dass der Aufbau der Zellwand bzw. des Zellmembrans bei den früher erwähnten Pilzstämmen, ihre Permeabilität (oder unter Umständen auch ihr Stoffwechsel) weitgehend verschieden sein kann von denen der später erwähnten Pilzarten. Die früher und später erwähnten Gruppen der Pilzstämme zeigen eine voneinander mehr oder weniger abweichende Sensibilität nicht nur gegenüber vielen Phenol-Derivaten, sondern auch gegen vielen Verbindungen von ganz anderer Struktur und verschiedenem Wirkungsmechanismus.

Auch dieser Umstand zeigt darauf, dass der Zellaufbau, die chemische Struktur ihrer Zellwand, bzw. Zellmembrans dieser zwei Gruppen der Pilze, und infolge dessen auch ihre Permeabilität, voneinander bedeutend abweichen kann; nur alle diese Umstände vorausgesetzt können wir ihre so verschiedenartige Empfindlichkeit gegenüber einer ganzen Anzahl von Verbindungen erklären (z.B. die Phenol-Derivate von Nr. F/2, 3, 4, 5, 6, 16, 17, 20, 23, 25, 55, 57, 58, 64, 68, 71, 87, 88, 89, 90, 94, 95, 102, 103, 104, 105, 106, 107, 120, 121, 122, 179, 225, 231, 240, 246, 247, 252, 254, 259, 260, usw.).

Wenn wir nun vergleichen die fungistatische Wirkung der untersuchten Phenol-Derivate, welche sie auf dieselben Pilzstämme auf flüssigem Serum-Maische-Nährboden und der auf festem Maische-Agar Kulturmedium ausüben, so erscheint es uns gleich auffallend, dass in den auf zwei verschiedenen Nährboden-Arten bekommenen Ergebnissen in zahlreichen Fällen recht grosse Unterschiede zu finden sind (so z.B. bei den Verbindungen von Nr. F/7, 15, 18, 19, 28, 74, 207, 208, 225, 246, usw.). Das gleiche lässt sich beobachten werden zahlreichen Verbindungen von anderer Struktur, die zu anderen Verbindungsgruppen gehören. Die Ursachen dieser Erscheinung sind physikalisch-chemische Problemen; ihre Erörterung ist nicht unsere Zielsetzung, so können wir diese hier übergehen.

Die Ergebnisse der auf flüssigem Maische-Nährboden unternommenen Untersuchungen über den Wirkungsmechanismus erwiesen, dass das Rinderserum, Lecithin, Lanolin, ja sogar auch das Sonnenblumenöl die auf das *Penicillium simplicissimum* ausgeübte fungistatische Wirkung zahlreicher Phenol-Derivate um 0,5–0,20 Teil ihrer ursprünglichen Wirkung abzuschwächen vermag; dagegen kann das Cholesterin ihre

fungistatische Wirkung nicht beeinflussen. Dabei kann das Serum die fungistatische Wirkung einiger Phenol-Derivate entweder durchaus nicht, die erwähnten anderen Stoffe auch nicht, oder nur in geringem Masse vermindern (z.B. die der Verbindungen von Nr. F/18, 19, 28, 29, 40, 71, 207, 211, 212).

Die auf das *Penicillium simplicissimum*, *Trichothecium roseum*, *Saccharomyces cerevisiae* auf festem Nährboden ausgeübte fungistatische Wirkung der untersuchten Phenol-Derivate (ausgenommen die des F/120) wird durch die Zugabe von 10% Rinderserum zum Nährboden (=etwa 0,7% Serum-Eiweiss-Gehalt im Kulturmedium) im Verhältnis zu den auf serumfreiem gewonnenen Ergebnissen gänzlich aufgehoben. Die Phenole werden vermutlich durch salzartige Bindungen an die Eiweisse geschlossen; diese Bindungen werden wahrscheinlich zwischen den basischen Gruppen der Eiweisse und den Hydroxyl-Radikalen von sauerem Charakter der Phenole gebildet. Ausser diesem Typ der Bindung sollen dabei noch zwischen den verschiedenen Substituenten der Phenole und den einzelnen Zentren der Eiweissmoleküle enstehenden Koordinative Bindungen unbedingt eine wichtige Rolle spielen. Die Eiweisse werden die fungistatische wirkung irgendeines Phenol-Derivates umso stärker vermindern, je stabiler der zu ihnen anknüpft ist.

Das Sonnenblumenöl und das Lanolin (das letzere bildet die Cholesterin-ester von gesättigten und ungesättigten Fettsäuren) können die fungistatische Wirkung der untersuchten Phenol-Derivate vermutlich dadurch vermindern, dass sie jene in sich selbst durchlösen und dadurch die ihren in der wässerigen Phase des Nährbodens erreichten Konzentrationen herabsetzen. Das Cholesterin, das im Nährboden (gegenüber dem in Form von Fetttropfchen anwesenden Sonnenblumenöl, Lanolin und Lecithin) in Kristall-Form anwesend war, vermochte wahrscheinlich die untersuchten Verbindungen nicht in sich selbst "hinüberzulösen", und so konnte auch ihre fungistatische Wirkung nicht beeinflussen. Der die fungistatische Wirkung der Phenole hemmenden Eigenschaft des Lanolins und Sonnenblumenöls liegen nach unserer Meinung bloss physikalische Vorgänge zugrunde. Von der gleichen Natur mag wohl

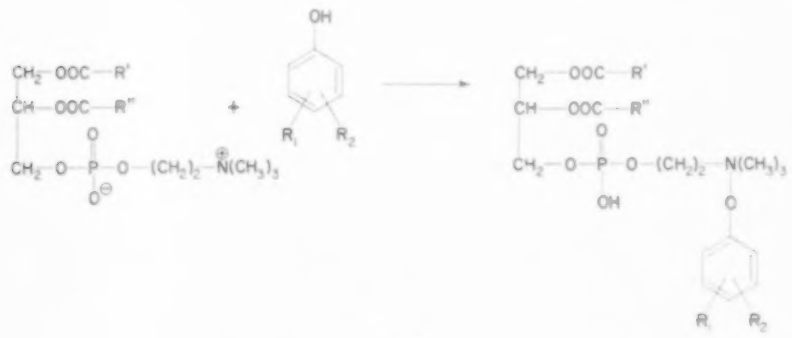

FIG. 1.

auch der wirkungshemmende Einfluss des Lecithins auf eine Anzahl der untersuchten Phenol-Derivate sein; es besteht jedoch auch die Möglichkeit, dass zwischen dem Lecithin und einigen Phenolen,—ausser der "Durchlösung" von rein physikalischer Natur—auch noch eine chemische Bindung zustande kommen kann auf die folgende Weise:

Es ist anzunehmen, dass in der Denaturierung der Zellwände und Fäden der Pilze durch Phenol-Derivate sowohl diese erwähnte Reaktion, als auch die damit verwandten Reaktionen (z.B. ihre, die mit Kephalin- und Sphingosin-artigen Lipoiden von Pilzen entstehenden Reaktionen) eine beträchtliche Rolle spielen; sie sind vermutlich—ausser der Eiweiss-Denaturierung—wichtige Komponente des biochemischen Wirkungsmechanismus der Phenol-Derivate von fungistatischer Wirkung.

Bei der Untersuchung der fungistatischen Wirkung der Phenol-Derivate auf *Penicillium simplicissimum* auf flüssigem Nährboden von pH = 4, 6 und 8 haben wir erfahren, dass die fungistatische Wirkung jener Verbindungen, deren Dissoziations-Konstante ungefähr die gleiche Grössenordnung besitzt als der des Phenols selbst (pK_a = 9,89), auf den Kulturmedien von den allen drei pH-Grössen sich auf der gleichen Stufe zeigte (z.B. die Verbindungen F/8, 18, 62, 71, 93, 109, 110). Dagegen zeigen jene Phenol-Derivate, bei denen der negative Logarithmus der Dissoziations-Konstante (=pK_a) unter 8 steht, also jene, welche mehrere "negativisierende" Substituente enthalten, *im allgemeinen* eine stärkere fungistatische Wirkung auf einem Maische-Nährboden von pH = 4 oder 6, als auf einem von pH = 8 (z.B. die Verbindungen F/20, 22, 23, 41, 53, 64). Für das abweichende Verhalten der Verbindungen Nr. F/32, 40, 42, 114, 115-obwohl ihr pK_a unter 8 steht-können wir zurzeit keine ausreichende Erklärung geben.

Obwohl die Phenol-Derivate, bei denen der negative Logarithmus der Dissoziations-Konstanten nierdrig ist ($pK_a < 8$), also jene, die *verhältnismässig* gut dissoziieren und demzufolge eine bedeutende Säuren-Eigenschaft besitzen, sind im allgemeinen wesentlich wirksamer, als jene von höherem (> 9) pK_a; jedoch werden wir auf Grund der vorhergehenden Behauptungen voraussetzen müssen, dass die "Wirkungsform" der Phenole das nicht dissoziierte ganze Molekül selbst bildet und nicht etwa das Phenolat-anion, was durch die Resultate unserer auf Kulturmedien von verschiedenem pH unternommenen Experimenten bestätigt wird.

Die Dissoziation der Phenole, besonders jener mit niedrigerem pK_a, besitzt nämlich bei pH = 8 einen höheren Grad, als bei pH = 6 oder 4; die Acidität des Nährbodens drängt die Dissoziation der Phenole zurück. Unsererseits erachten es für wahrscheinlich, dass die Phenol-Derivate in nicht disszoziierte liposolubiler Form in die Zellwände und Zellmembran der Sporen und Fäden der Pilze diffundieren, aber dort reagiert schon ihre dissoziierte Form mit den Eiweissen und Lecithinen derselben, oder mit ihren auch andere basische Zentren enthaltenden lebenswichtigen Bestandteilen.

Aus den Zusammenhängen der fungistatischen Wirkung der Phenol-Derivate mit ihrem chemischen Aufbau lässt sich der Schluss ziehen, dass nur jene Phenol-Derivate eine bedeutende fungistatische Wirkung besitzen, welche folgende physikalisch-chemische und strukturelle Eigenschaften besitzen:

1. *Ein hochgradiger Verteilungs-Quotient zwischen Lipoid und Wasser*

Jene Phenol-Derivate, bei denen der Verteilungsquotient zwischen Lipoid und Wasser allzu hoch ist (praktisch ∞), erweisen sich wirkungslos (z.B. F/47, 51, 52, 69, 117, 118, 163, 164, 180, 181, 182, 185, 187, usw.); diejenigen, bei denen dieser Quotient sehr niedrig ist (z.B. die Sulfonsäuren und ihre Salze), sind auch unwirksam (z.B. die F/56, 59, 60, 63, 166–174).

Der Verteilungsquotient zwischen Lipoid und Wasser der Verbindungen von Nr. F/55, 57, 58, 120, 121, 122 ist zwar niedrig, diese sind aber doch fungistatisch wirksam; deren Wirkungsmechanismus ist aber von ganz anderer Natur, wie der von den anderen Phenol-Derivaten.

2. *Molekülоberfläche von entsprechender Grösse oder Alkyl-Substituent von entprechender Länge*

Diese sind notwendig, um einerseits den Verteilungsquotient zwischen Lipoid und Wasser zu erhöhen, anderseits aber, damit die Adsorption der Verbindungen and die Lipoproteide der Zellwand oder des Zellmembrans der Pilzsporen und Pilzfäden auf einer entsprechenden Fläche von optimaler Grösse verwirklicht werde. Wenn die Bindung irgendeines Phenol-Derivates an die Pilzelemente nicht nur durch phenolische Hydroxyl-Radikale mittels lauter chemischer Kräfte erfolgt, sondern auch auf dem Wege physikalisch-chemischer Adsorption, dann ist die Affinität einer solchen Verbindung an die Pilzelemente, andererseits aber die Stabilität der schon entstandenen Bindungen beträchtlich hochgradiger, also ohne die Mitwirkung derselben.

Die Oberfläche des Benzolringes betrug 17,4Å² die des Naphtalinringes 27,9Å² die des Anthrazenringes aber 38,5Å².[61]

Es muss eine optimale Oberfläche geben (ungefähr von gleicher Grösse wie die des Naphtalins), die das Molekül erreichen soll, um fungistatisch wirksam zu werden; andererseits darf es aber auch diese Grösse der Oberfläche nicht bedeutend überschreiten.

Die in die Phenole eingeführten Alkyl-, Alkyloxy- und Aryl-Substituenten können *nicht nur* durch die Erhöhung des Verteilungsquotienten zwischen Lipoid und Wasser die fungistatische Wirkung steigern, sondern auch dadurch, dass sie infolge der Van der Waalsschen Kräfte intermolekular aneinander geschlossen die Phenol-Derivate in Form mit "micellaler" Struktur verwandeln, welche Micellen, d.h. Molekül-Assoziaten eine Affinität an die Pilzelemente—und dadurch auch die fungistatische Wirkung der zur Assoziation geneigten Phenol-Derivate im allgemeinen—in bedeutend grösserem Masse besitzen, als die sonst nicht assoziierten einzelnen Moleküle hätten (z.B. das F/8, 10–15 gegenüber F/1; F/2 gegenüber F/56 und 60; F/87–90 gegen F/86; F/92–95 gegen das F/91, usw.). Allzu lange Alkyl-Substituenten, oder die gemeinsame Anwesenheit von Alkyl-Gruppen zwar von mittlerer Länge und dabei aber noch *mehreren Halogen-Atomen* in dem Molekül (siehe die Verbindungen von Nr. F/68, 69, 99, 100) erhöhen beträchtlich den Verteilungquotienten zwischen Lipoid und Wasser der Phenole; deshalb sind die Phenol-Derivate mit solchen Substituenten, bzw. von solcher Struktur wirkungslos.

3. *Eine zur Ionisation geeignete freie phenolische Hydroxyl-Gruppe*

Die Bindung der Phenol-Derivate an die Pilzelemente erfolgt hauptsächlich durch die phenolische Hydroxyl-Gruppe; so besitzt also dieses Radikal mit Rücksicht auf die fungistatische Wirkung der Verbindungen dieser Klasse eine hervorragende Bedeutung.

Dabei muss man aber auch feststellen, dass es Ausnahmen von dieser Regel gibt. Z.B. eine fungistatische Wirkung haben die O-Acetyl-phenole und auch einige Phenol-äther (siehe die Angaben der Tabellen).

Aus diesem lässt sich der Schluss ziehen, dass die Bindung zwischen den Eiweissen, Lipoiden, Lipoproteiden der Pilzsporen und Pilzfäden, sowie den verschiedenen Phenol-Derivaten *nicht nur* durch die Salzbildung mittels Hydroxyl-Radikale entstehen kann, sondern- durch den Oxygen-Atom!—auch mit der Vermittlung von Neben-Valenzen, Koordinations-Bindungen von *Van der Waal'schen Kräften* zugleich erfolgt.

Der negative Logarithmus der Dissoziations-Konstante (= pK_a) der Phenol-Derivate von freien Hydroxyl-Gruppen vermindert sich durch Halogen-Substitution. Ihre fungistatische Wirkung erhöht sich—innerhalb gewisser Grenzen—damit parallel, aber nur in dem Falle, wenn diese durch andere strukturelle Faktoren nicht verhindert wird.

Die Dissoziation der phenolischen Hydroxyl-Gruppen steigernde Wirkung der in die Moleküle der Phenole eingeführten Halogen-Atome können wir durch die unteren Formeln veranschaulichen:

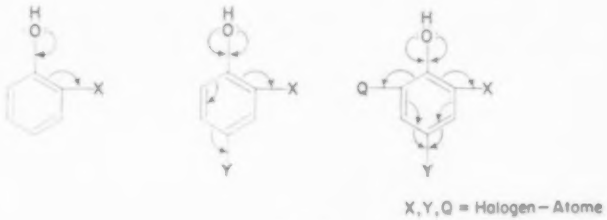

FIG. 2.

Aus diesen Formeln ersieht man, dass die X, Y, Q Substituenten durch ihre elektrophyle Eigenschaft die Elektronen-Dichte um das phenolische Oxygen-Atom vermindern, infolge dessen vermag das Phenol leichter das ihm gehörende Proton als H^+-Ion abzugeben, und das umso leichter, je mehr elektrophyle Substituenten (= X, Y, Q) das Molekül enthält; weil die Elektronen-Dichte um das Oxygen-Atom wird ja damit umso geringer.

Die zwei Halogen-Atome enthaltenden Phenole haben meist eine stärkere fungistatische Wirkung, als die nur mit einem Halogen-Atom; die mit drei Halogen-Atomen sind noch wirksamer.

Jene Phenol-Derivate, welche bei ihrer Dissoziation ihre elektrische Ladung nicht auf ihrem phenolischen Oxygen-Atom tragen, bleiben gänzlich oder beinahe ohne Wirkung—wie z.B. die Phenol-karbonsäuren (F/75–85). Durch Esterisation ihrer saueren Karboxyl-Gruppe (im Falle, wenn der zur Esterisation gebrauchte Alkohol drei bis sechs Kohlenstoff-Atome enthält!) kommen durch sie wirksame Verbindungen zustande; die infolge der Esterisation entstandenen Phenol-karbonsäure-ester benehmen sich schon nämlich als lipophyle Substituenten besitzende Phenole, bei deren Dissoziation nicht mehr Phenol-karboxylat, sondern Phenolat-anion entsteht (F/87–90, 92–98, 101–107). Für wirkungslos gelten auch die basischen Nitrogen-Atom enthaltenden Phenol-Derivate, denn die säurige Dissoziation ihrer phenolischen Hydroxyl-Gruppe wird durch die basische Dissoziation ihres Nitrogen-Atoms zurückgedrängt, bzw. ihre bei der Dissoziation entstehenden positiven und negativen Ladungen heben sich gänzlich oder teilweise gegenseitig auf (z.B. F/123, 126–135 usw.). Eine Ausnahme davon bilden die Verbindungen von Nr. F/120, 121

und 122, deren Wirkungsmechanismus vermutlicherweise weitgehend von dem der meisten Phenol-Derivate abweichend ist. Diese Verbindungen (und jene von Nr. F/55, 57, 58, gleichfalls) können ihre fungistatische Wirkung wahrscheinlich auf dem Wege ausüben, dass sie in dem Nährboden zu Chinonimine (bzw. zu Chinone) oxidiert werden und diese Oxidationsprodukte werden erst zu den eigentlichen Agenten von fungistatischer Wirkung: sie hemmen nämlich die Tätigkeit einiger lebeswichtigen Sulfhydryl-Enzyme der Zellen von Pilzen. Sie üben also ihre Wirkung—nach unserer Voraussetzung—abweichend von den meisten Phenol-Derivaten, nicht auf die Zellwand, bzw. auf das Zellmembran der Pilze, sondern auf intrazellulären Stoffwechsel-Vorgänge aus.

Die Ergebnisse unserer Forschungen beweisen, dass die fungistatische Wirkung der unsererseits in vitro am wirksamsten gefundenen Phenol-Derivate durch das Serum (und vermutlich auch duch andere Eiweiss-arten), sowie durch verschiedene Lipoide bedeutend vermindert wird. Diese Erscheinung lässt uns darauf schliessen, dass das Wirkungsmechanismus der Phenol-Derivate *nicht ausschliesslich* die Hemmung *irgendeines spezifischen biochemischen Vorganges* der Pilze zugrunde liegt, sondern ihre fungistatische Wirkung durch aspezifische Denaturierung der Zellwand, bzw. des Zellmembrans erreicht wird. Diese Wirkung beschränkt sich freilich nicht nur auf die verschiedenen Pilzarten, sondern mag überhaupt für alle lebende Zellen gelten (deshalb ist sie ja eben aspezifisch!).

ZUSAMMENFASSUNG

Verfasser hat die fungistatische Wirkung von 260 Phenol-Derivaten untersucht, die sie auf flüssigem Serum-Maische Nährboden, sowie auf festem Maische-Agar Nährboden ausübten, und den Wirkungsmechanismus derjenigen studiert, welche er in seiner Versuchen am wirksamsten gefunden hatte.

Er behandelte zugleich die Zusammenhänge, welche zwischen der *in vitro* ausgeübten fungistatischen Wirkung der Phenol-Derivate und deren chemischem Aufbau bestehen. Herausgegangen von der Beeinflussbarkeit ihrer fungistatischen Wirkung durch Serum-Eiweisse, Lecithin, Lanolin, Cholesterin uns Sonnenblumenöl suchte er Schlüsse bezüglich des Wirkungsmechanismus der Phenol-Derivate zu ziehen.

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Biochemical Pharmacology, 1960, Vol. 5, pp. 20–26. Pergamon Press Ltd., Printed in Great Britain.

THE ACTION OF CHLORPROMAZINE ON YEAST HEXOKINASE

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(*Received* 7 *August* 1959; *revised* 18 *March* 1960)

Abstract—The effect of chlorpromazine on yeast hexokinase was investigated in a reaction system in which the activity of the kinase enzyme, coupled to glucose-6-phosphate dehydrogenase, was determined by measuring spectrophotometrically the reduction of TPN. Hexokinase activity, either in the presence or absence of chlorpromazine, showed great sensitivity to the magnesium–ATP ratio.

Concentrations of chlorpromazine from $8{\cdot}5 \times 10^{-6}$M to $6{\cdot}8 \times 10^{-5}$M either greatly activated or inhibited hexokinase activity, depending on the level of magnesium–ATP in the system.

Implications of the observed effects of CPZ on hexokinase are discussed in relation to some of the pharmacological activities of the drug.

INTRODUCTION

Hypothermia and hyperglycemia have been reported in laboratory animals[1–3] and human subjects[4–5] receiving chlorpromazine (CPZ).* Many studies have shown that CPZ inhibits oxygen uptake in various tissues,[6–7] but little evidence of a direct effect of CPZ on the substrate phosphorylation reactions involved in glucose utilization has been discerned. Various workers have shown that in mitochondrial preparations CPZ uncouples oxidative phosphorylation[8–11] and inhibits the activity of ATPase,[8,11,12] cytochrome oxidase,[8,12] and reactions coupled to the oxidation of DPNH.[13] Thus, it would seem possible that any effect of CPZ on anaerobic carbohydrate metabolism might be through action of the drug on enzyme systems associated with phosphorylation reactions.

Earlier work in this laboratory showed that administration of CPZ *in vivo* decreased the formation of pyridoxal-5-phosphate from ATP and pyridoxal in brain homogenates from normal and vitamin B_6-deficient rats.[14] These results were interpreted as an indication that CPZ prevented the transfer of phosphate from ATP to pyridoxal by inhibiting the action of the enzyme pyridoxal kinase. Since the pyridoxal kinase system resembles the phosphokinase reactions occurring in anaerobic glycolysis, studies were undertaken to test the effect of CPZ on a glycolytic kinase.

Although the work previously done with CPZ has principally involved various mammalian cell preparations and enzymes, some work has been reported in which glycolytic enzymes from yeast were used.[9] These preparations are readily available in purified form and offer the further advantage of being well characterized. For these

* The following abbreviations are used: chlorpromazine-HCl (10-[3-dimethylaminopropyl]-2-chlorophenothiazine-HCl) (SKF no. 2601-A), CPZ; adenosine-5′-triphosphate, ATP; diphosphopyridinenucleotide, oxidized and reduced form, DPN and DPNH, respectively; triphosphopyridine nucleotide, oxidized and reduced form, TPN and TPNH, respectively; dinitrophenol, DNP.

reasons the present study was carried out using yeast hexokinase rather than a mammalian preparation.

EXPERIMENTAL METHODS

Determination of enzyme activity

The activity of hexokinase was measured by coupling the kinase reaction to glucose-6-phosphate dehydrogenase (*Zwischenferment*) and determining spectrophotometrically the change in absorbency at 340 mμ due to the reduction of TPN:

glucose + ATP → glucose-6-phosphate + TPN → 6-phosphoglucono-δ-lactone + TPNH

The velocity of the initial phase of the reaction (during the first 60 or 90 sec) was used as an index of hexokinase activity. In this system the concentration of the *Zwischenferment* was maintained in excess over the hexokinase concentration, thereby insuring that the rate of the phosphorylation reaction limited the rate of TPN reduction (Fig. 1).

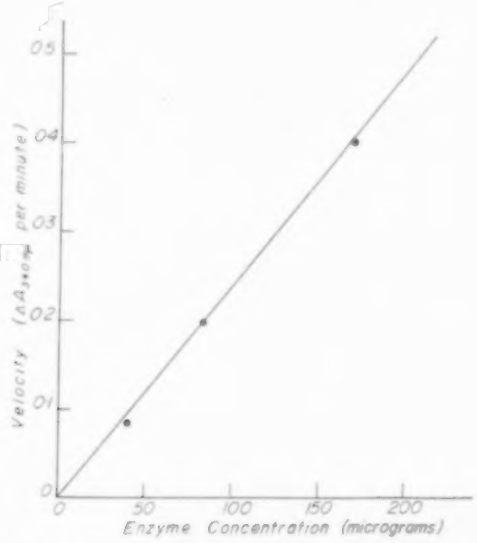


FIG. 1. Effect of enzyme concentration on the rate of TPN reduction.

All reagents were obtained commercially.* The reaction system contained: tris-(hydroxymethyl)aminomethane, 0·05 M, pH 7·5; potassium dihydrogenphosphate 0·005 M; crystalline yeast hexokinase, 0·04 mg; *Zwischenferment*, 0·75 mg; glucose, 0·1 M; TPN, 2×10^{-4}M; ATP and magnesium chloride. The final reaction volume in the cuvettes was 3·0 ml. In order to determine the effect of CPZ, various concentrations of the drug were added to the enzyme solution. After incubation for 15–20 min at room temperature, 0·2 ml of the enzyme–CPZ solutions were added to the reaction system containing all components except ATP. The reaction was initiated by the addition of ATP. The concentrations of CPZ given in the text are the final concentrations of the drug in the reaction systems.

* Hexokinase (crystalline), Pabst laboratories. Glucose-6-PO_4 dehydrogenase, Type II, Sigma Chemical Co., St. Louis, Mo.

The reaction in the absence of CPZ was first studied to determine the experimental conditions to be used in the studies with CPZ and as a check on the characteristics of the enzyme preparation used. With glucose (0·1 M) and $MgCl_2$ (1×10^{-3}M) held constant, ATP was a strong inhibitor of the reaction when present in excess of $2{\cdot}5 \times 10^{-4}$M (Fig. 2). When glucose and ATP were held constant, inhibition was observed if the magnesium concentration greatly exceeded the initial ATP concentration (Fig. 3). A number of reports indicate molar ratios of Mg–ATP near 1:1 as the true sub-

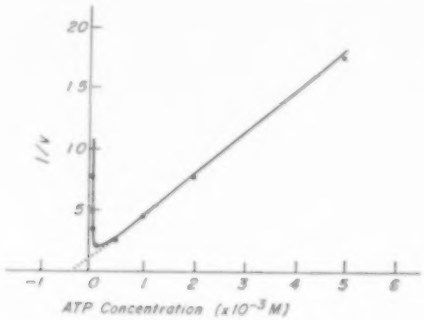


Fig. 2. Effect of ATP concentration on initial reaction rates in the presence of 1×10^{-3}M $MgCl_2$.

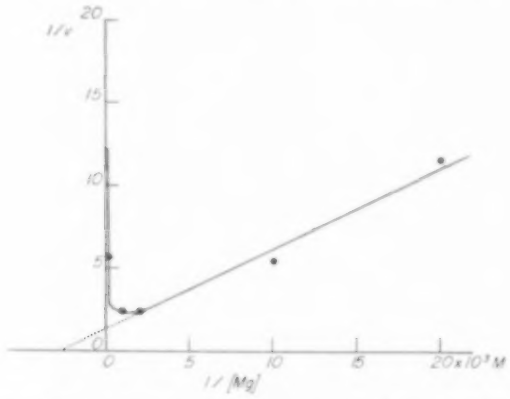


Fig. 3. Reciprocal plot of the effect of Mg^{2+} concentration on initial reaction rates in the presence of 1×10^{-3}M ATP.

strate species of phosphokinase reactions;[15–17] a plot of enzyme activity against Mg–ATP (1:1) concentration is given in Fig. 4. This 1:1 Mg–ATP ratio was used in all subsequent studies.

The effect of CPZ on the initial enzyme rates were assessed in a hexokinase system in which half-maximum activity was obtained at a Mg–ATP concentration of $2{\cdot}5 \times 10^{-4}$M The maximum initial velocity was found to correspond to a change in absorbancy of 0·100 units/min.

Hexokinase activities at various concentrations of Mg–ATP were determined in the presence of four levels of CPZ: $8{\cdot}5 \times 10^{-6}$M, $1{\cdot}75 \times 10^{-5}$M, $3{\cdot}5 \times 10^{-5}$M, and $6{\cdot}8 \times 10^{-5}$M (Figs. 5–8). The enzyme activities at the various Mg–ATP concentrations in the absence of CPZ served as control rates.

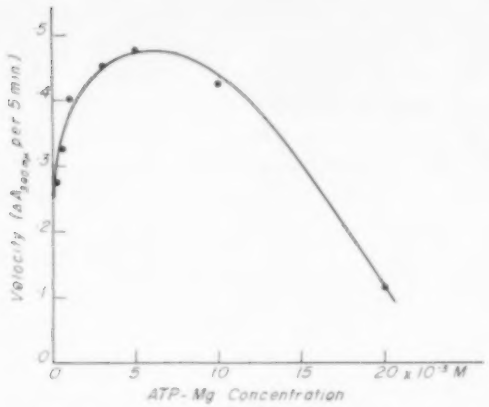
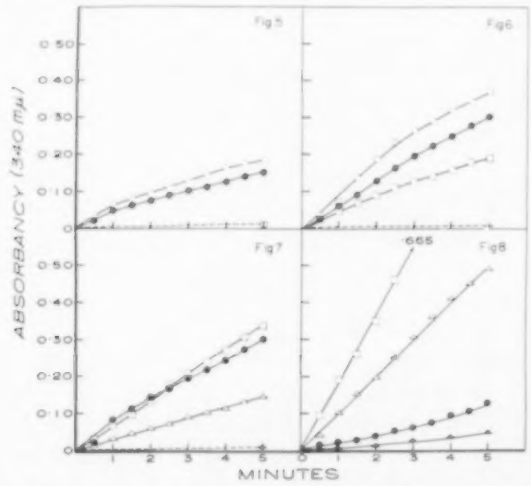


FIG. 4. Effect of ATP–Mg concentration at a 1 : 1 molar ratio on reaction rates.

RESULTS

CPZ acted as either an activator or inhibitor of hexokinase, depending on the concentration of the drug in relation to the concentration of Mg–ATP present. At the lowest concentration of CPZ ($8{\cdot}5 \times 10^{-6}$M), a slight activating effect on hexokinase was observed with a Mg–ATP concentration of 1×10^{-4}M, while at a higher concentration of Mg–ATP (5×10^{-4}M), a 35 per cent increase over the control rate of hexokinase activity was observed.

In the presence of $1{\cdot}75 \times 10^{-5}$M CPZ, no measurable hexokinase activity was detected with a Mg–ATP concentration of 1×10^{-4}M; however, by increasing the



FIGS. 5–8. The effect of chlorpromazine–HCl on yeast hexokinase. Mg-ATP (1 : 1 M). Conc.: Fig. 5, 1×10^{-4} M; Fig. 6, 5×10^{-4} M; Fig. 7, 1×10^{-3} M; Fig. 8, 2×10^{-2} M. Chlorpromazine-HCl concentration:

(●) none (control); (○) $8{\cdot}5 \times 10^{-6}$ M;
(□) $1{\cdot}75 \times 10^{-5}$ M; (△) $3{\cdot}5 \times 10^{-5}$ M;
(▲) $6{\cdot}8 \times 10^{-5}$ M.

Mg–ATP concentration, it was possible to restore the kinase activity to 70 per cent of the control rate with 5×10^{-4}M Mg–ATP and to approximately 100 per cent of the control rate with 1×10^{-3}M Mg–ATP. When the Mg–ATP concentration was raised to 2×10^{-2}M, the presence of $1{\cdot}75 \times 10^{-5}$M CPZ increased the enzyme activity 4·5 times over that of the control rate observed at this level of Mg–ATP.

At $3{\cdot}5 \times 10^{-5}$M CPZ, no measurable hexokinase activity was found with a Mg–ATP concentration of 5×10^{-4}M; however, enzyme activity could be restored by higher concentrations of Mg–ATP. A concentration of 1×10^{-3}M Mg–ATP restored

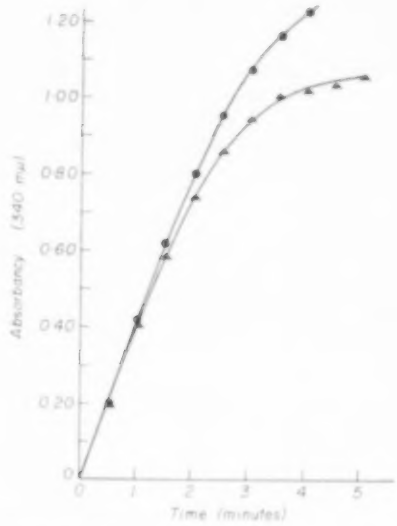


Fig. 9. Effect of CPZ on glucose-6-phosphate dehydrogenase.
(●) no CPZ; (▲) $6{\cdot}8 \times 10^{-5}$ M CPZ.

the enzyme activity to 30 per cent of the control rate. However, in the presence of this level of CPZ, a twofold increase in rate over that of control was observed when the Mg–ATP concentration was raised to 2×10^{-2}M.

At the highest concentration of CPZ used in these experiments ($6{\cdot}8 \times 10^{-5}$M) no hexokinase activity was found with concentrations of Mg–ATP up to 5×10^{-3}M (not shown in figure). An increase of Mg–ATP to 2×10^{-2}M restored the rate to only to 10 per cent of that of the control.

Since the reaction rate in the presence of the highest level of CPZ used in these experiments ($6{\cdot}8 \times 10^{-5}$M) was restored to only 10 per cent of the control rate by 2×10^{-2}M Mg–ATP, it was important to determine whether the almost complete loss of enzyme activity observed at this concentration of CPZ was the result of the action of the drug on hexokinase or the result of a rate-limiting inhibition of the auxiliary enzyme. To determine the activity of the *Zwischenferment* alone, an excess of glucose-6-phosphate was substituted for Mg–ATP in the reaction system. The rate of TPN-reduction, under these conditions, indicated that the *Zwischenferment* activity, in the presence of $6{\cdot}8 \times 10^{-5}$M CPZ, was inhibited by only 20 per cent, and therefore did not limit the rate of the reaction sequence (Fig. 9).

DISCUSSION

These studies suggest that small variations in the concentration of CPZ can either activate or inhibit activity of an isolated hexokinase system. When the Mg–ATP concentration is low in comparison to the concentration of CPZ, the drug acts essentially as an inhibitor of this kinase system and prevents the utilization of ATP; whereas, at high concentrations of Mg–ATP relative to the concentration of CPZ, the drug functions as an activator, increasing the rate of utilization of ATP.

This apparent dual activity of CPZ has been observed in more complex enzyme systems and in tissue preparations, in which stimulatory effects have been observed at low concentrations of CPZ, while at higher concentrations of the drug, depressed activity was obtained.[8, 11, 18, 19] As an example, in rat brain homogenates, glutamine synthesis was stimulated by low concentrations of CPZ, while at only slightly higher concentrations the drug abruptly inhibited glutamine formation by nearly 100 per cent.[19] A biphasic action of CPZ on the DNP-stimulated ATPase reaction in rat-liver mitochondria has recently been reported by Löw,[20] who, in addition, found that this effect is apparently not restricted to CPZ; a number of chemically-unrelated substances, such as atebrin, acriflavin and antimycin A, also exhibit a similar effect on DNP-stimulated ATPase.[21] It seems pertinent to our observations in the isolated enzyme system that a similar dual effect has been observed in whole organisms. Low concentrations of CPZ have been shown to stimulate the growth of *L. acidophilus*, whereas high concentrations of the drug inhibited growth of this organism,[22] and growth of vitamin B_6-deficient rats has been stimulated by low doses of CPZ, while higher levels either failed to stimulate or depressed growth.[23]

The observed effects of CPZ on yeast hexokinase reported here resemble those described by Yanagawa[24] who reported stimulation of rat brain hexokinase by low levels of CPZ but inhibition by higher levels, and by Allenby and Collier[25] who found various phenothiazines to be potent inhibitors of rat brain hexokinase. However, there are several reported studies of the effect of CPZ on anaerobic glycolysis which differ from these results. Löw[26] investigated the effect of CPZ on yeast hexokinase by determining the decrease in acid-labile phosphate and observed no difference in ATP utilization in the presence of CPZ. Bernsohn *et al.*[9] reported that 1×10^{-3}M CPZ had no effect on anaerobic glycolysis in rat brain homogenates. However, under aerobic conditions he found that CPZ greatly depressed the utilization of glucose by the homogenates but not by isolated yeast hexokinase. A possible explanation for the differences between the results of the above investigators[9, 26] and those reported here may be found in differences in concentrations and ratios of ATP and magnesium chosen and in the methods used by the various investigators. Indeed, one factor which seems to stand out in the work reported here is that CPZ appears to affect the "dissociation" or "binding" constants of the enzyme for Mg and ATP, which have already been shown to be interdependent cofactors of hexokinase. Therefore, any conclusions projected from observed results in this system are closely dependent on the choice of experimental conditions.

The results presented in this paper suggest that the often observed hyperglycemic effect following the administration of CPZ should be accompanied by an accumulation of ATP if its utilization were decreased. This contention is supported by the studies of Grenell[27] who found increased levels of ATP in the lungs and cerebral cortex of CPZ-treated rats. In nervous tissues, the greatest increase in ATP was observed in the

thalamus–hypothalamus area of the brain. These increased tissue levels of ATP parallel the distribution patterns of tissue-CPZ reported by other investigators.[28, 29]

These observations are not sufficient to permit a definitive conclusion concerning the mechanism of action of the drug; however, our data seem to implicate interference with utilization of ATP as a fundamental metabolic effect of chlorpromazine administration.

SUMMARY

Chlorpromazine may either inhibit or activate the utilization of ATP by an isolated system of yeast hexokinase. This biphasic action of the drug appears to be a function of the relative concentrations of the drug and the Mg-ATP substrate. The ability of chlorpromazine either to stimulate or to inhibit the utilization of ATP suggests that it may function as a metabolic regulator. The possibility of attributing some of the pharmacologic properties of the drug to its apparent regulatory action is discussed.

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Biochemical Pharmacology, 1960, Vol. 5, pp. 27–29. Pergamon Press Ltd., Printed in Great Britain.

THE COMPARATIVE METABOLISM OF MYLERAN-^{35}S IN THE RAT, MOUSE AND RABBIT

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(*Received* 18 *February* 1960)

Abstract—The fate of Myleran-^{35}S has been examined in three species. The rat and mouse excretes ^{35}S-labelled materials in similar proportions, whereas the rabbit excretes only methane sulphonic acid-^{35}S. The rabbit appears to be deficient in the urinary component which combines with methane sulphonic acid in the rat and mouse.

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Chin-Tsu Peng[1] has shown that after injection into rats of Myleran-^{35}S (approx. 4 mg/kg in propylene glycol) the urinary metabolites (95–96 per cent of the dose after 32 hr) consisted mainly of methane sulphonic acid, some Myleran and other unidentified components. The amount of Myleran excreted unchanged within 24 hr was approximately 6 per cent of the dose. More recently, with the same species, Trams *et al.*[2] have shown that, following a smaller dose (2 mg/kg intraperitoneally, solvent assumed to be 10 per cent acetone), 60 per cent of the radioactive dose was excreted in 24 hr. Of this activity, 62–68 per cent was methane sulphonic acid, 25–30 per cent Myleran, 0·03–0·04 per cent sulphate and 5–8 per cent an unidentified component.

We have made some preliminary observations on the fate of Myleran-^{35}S in three species at the same dose level (10 mg/kg) used by us in earlier studies.[3] The compound, prepared according to the method of Chin-Tsu Peng,[1] with a specific activity of 8·6 mc/m-mole, was given intraperitoneally in arachis oil. The radioactivity present in urine and plasma was determined using 0·02 ml samples dried on aluminium trays with appropriate corrections for absorption.

In the rat, 50 per cent of the administered radioactive dose was excreted in the urine during the first 48 hr. The radioactive metabolites were separated by single-dimension paper chromatography (Fig. 1a) using two solvent systems, *n*-butanol:dioxan:2N ammonia (4:1:5) and *n*-butanol:2N acetic acid (1:1). Methane sulphonic acid and Myleran were identified whilst two components remained unidentified; one of these, (U1) lies very close to the methane sulphonic acid spot and the other (U2) is situated ahead of Myleran. Table 1 gives the R_f values of these metabolites in the two solvent systems. The radioactive material present in the 24 hr urine sample was mainly methane sulphonic acid and U1; these are not completely separated but autoradiographs suggest that approximately equal amounts were present. Only small amounts of injected Myleran (5 per cent) and U2 (2 per cent) were found. The plasma radioactivity reached a maximum level (6 per cent of the dose) approximately 3 hr after administration (Table 2) and consisted chiefly of unchanged Myleran (90 per cent) and methane sulphonic acid (8–10 per cent). No U2 was detected.

In the mouse, about 60 per cent of the radioactive dose appeared in the 24 hr urine sample. The relative amount of U1 was higher than in the rat, but the other three components found in the rat were also present in this species (Fig. 1b). The blood radioactivity reached a level of 1·6 per cent of the dose 1 hr after administration, 50–60 per cent being Myleran and the remainder methane sulphonic acid.

Table 1. Radioactive components of urine 0–24 hr after administration of Myleran-^{35}S

	R_f values		Percentage distribution of radio-activity in urine		
Metabolite	BDN*	BA†	Rat	Mouse	Rabbit
Sulphate	origin	origin	—	—	—
$MeSO_2OH$	0·16	0·12	90	91	100
U1	0·11	0·18			—
Myleran	0·85	0·86	5	4	—
U2	0·98	0·93	2	3	—

* *n*-Butanol, dioxan, ammonia.
† *n*-Butanol, acetic acid.

The level of radioactivity in the plasma 2½ hr after injection into a rabbit was 1·4 per cent of the dose; of this 20–30 per cent was unchanged Myleran, whereas methane sulphonic acid accounted for most of the remainder (Table 2). In 24 hr, 30 per cent of the radioactive dose was excreted, entirely as methane sulphonic acid (Fig. 1c). *In vitro* tests were carried out to determine the stability of Myleran in plasma, urine and buffered solution. Labelled Myleran was dissolved in urine and plasma from each species (approx. 100 μg/ml) as well as in phosphate buffer at pH 8·0. In each case Myleran hydrolysed with a half-life of 11–12 hr at 37 °C. In urine and plasma from the

Table 2. Radioactive components of plasma 2–4 hr after injection of Myleran-^{35}S

Metabolite	Rat	Mouse	Rabbit
Sulphate	<1	<1	<1
$MeSO_2OH$	8	40	60–70
U1			
Myleran	90	50–60	20–30
U2	—	—	—

The percentage distribution of radioactive components of plasma following administration of Myleran-^{35}S, 2–4 hr after injection.

rat and mouse U1 and U2 were present. There was no evidence of either component in the rabbit urine or in buffer. The unknown U1 may be a product of reaction of methane sulphonic acid with a urinary constituent, as suggested by Peng,[1] but this latter must be lacking in the rabbit. These results also suggest that protracted methods for the extraction of the metabolites e.g. ether extraction in a Soxhlet apparatus over

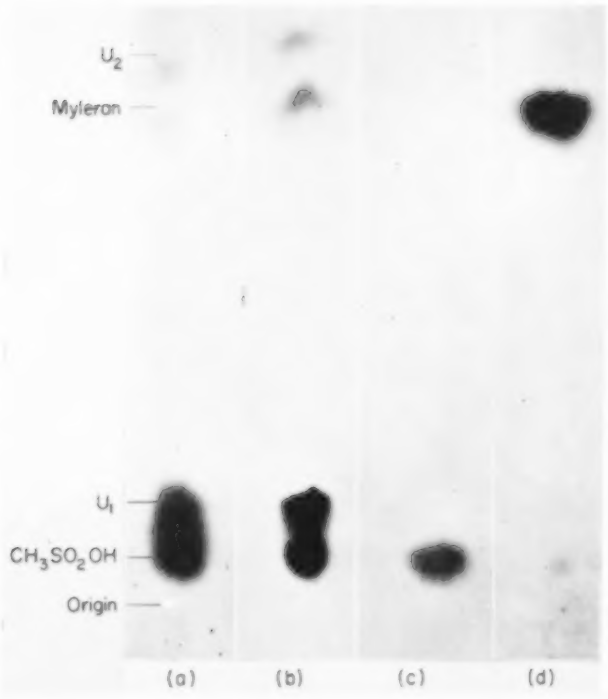

FIG. 1. Radioautographs of chromatograms indicating the 24 hr urinary components in three species after administration of Myleran-^{35}S. Solvent system, *n*-butanol:2N acetic acid (1:1). (a) Rat. (b) Mouse. (c) Rabbit. (d) Myleran alone.

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several hours,[2] should be avoided. Myleran alone was stable under the chromatographic conditions employed forming a single spot (Fig. 1(d)).

In summary, methane sulphonic acid was the only material excreted in the urine of a rabbit given ^{35}S-labelled Myleran. Rat and mouse urine contained, in addition to methane sulphonic acid and a little Myleran, two other unidentified components (U1 and U2) which appear to be produced by reaction of labelled material with a substance not present in the rabbit.

More detailed studies of the fate of sulphonoxy alkane groups in similar compounds are in progress.

Acknowledgement—The authors are indebted to Mr. J. Morrison for his technical assistance. The work was supported by a grant from the British Empire Cancer Campaign.

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ROUTES OF METABOLISM OF [^{36}Cl] RING-SUBSTITUTED MONOCHLOROACETANILIDES*†

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Abstract—The excreted monohydroxy and deacetylated metabolites of 2-, 3- and 4-[^{36}Cl]chloroacetanilide in the urine of rats were determined. The extent of *ortho*-hydroxylation (relative to the amino group) decreased in the same order as the relative toxicities and antipyretic activities of the administered drugs: 4- 3- 2-. Neither total hydroxylation nor *para*-hydroxylation followed this order. Deacetylation of the parent compound increased as the toxicity and antipyretic activity decreased. Conjugation of the hydroxylated metabolites as etheral sulfates and glucuronides was observed.

POSSIBLE relationships between metabolism and carcinogenicity of aromatic amines have been suggested by several investigators[1, 2] and the importance of the *o*-hydroxylated metabolites has been discussed in some detail.[3] These studies on the metabolism of carcinogenic amines have been extended also to simpler amines,[4] and it is possible that a systematic study with compounds of less complicated structure than the carcinogens may offer a clue to the problem of the metabolism of aromatic amines as related to physiological response.

It has been established that the analgesic and antipyretic drug, acetanilide, is metabolized by deacetylation, hydroxylation and conjugation as glucuronides or ethereal sulfates. Studies *in vitro* by Bray *et al.*[5] indicate that substitution in the various positions in acetanilide affects the extent of deacetylation markedly. Ring substitutions would also be expected to affect the position and extent of hydroxylation. In turn, such differences in hydroxylation and deacetylation might influence certain pharmacological properties of the compound. In fact, studies by Argus *et al.*[6] showed that the position of chloro-substitution in the ring altered the toxicity and antipyretic activity of acetanilide. This information suggested that the isomeric chloroacetanilides might be of value in studying the relationship between metabolism and these biological activities. The use of chlorine as the substituent in the aromatic ring of acetanilide gives the added advantage of permitting the use of the radioactive isotope, ^{36}Cl, as a tracer in inverse isotope dilution experiments for identifying metabolites.

The present study describes the quantitative determination or identification of metabolites of 2-, 3- and 4-[^{36}Cl]chloroacetanilide in the urine of rats receiving these compounds. The possible relationship between routes of metabolism and observed differences in the biological effects of the administered compounds is discussed.

* This work was supported by research grant C-1356 from the National Cancer Institute, Public Health Service.

† Taken from a dissertation by M. Newell presented to the University of Florida, June 1958, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

EXPERIMENTAL

Materials

Melting points of all compounds are uncorrected.

Radioactive compounds. 4-[^{36}Cl]Chloroacetanilide melting at 175–177 °C and having a specific activity of $3{\cdot}4 \times 10^4$ counts/min per mg was prepared according to Gryder *et al.*[7] The synthesis of 3-[^{36}Cl]chloroacetanilide (m.p. 73 °C; specific activity $1{\cdot}5 \times 10^4$ counts/min per mg) and 2-[^{36}Cl]chloroacetanilide (m.p. 88 °C; specific activity $2{\cdot}1 \times 10^4$ counts/min per mg) were described previously.[8]

Metabolites of 4-chloroacetanilide. 4-Chloroaniline and 4-chloroacetanilide (Eastman) were recrystallized from 50% aqueous ethanol to sharp melting points of 72 °C and 178 °C, respectively, in agreement with the literature.[9, 10] 4-Chloro-2-hydroxyaniline was prepared by the method of Hodgson and Kershaw.[11] Acetylation of this compound in benzene with equimolar portions of acetic anhydride yielded 4-chloro-2-hydroxyacetanilide, m.p. 190°.

Metabolites of 3-chloroacetanilide. 3-Chloroaniline (Eastman) was purified by distillation; the fraction distilling at 226–227 °C at atmospheric pressure was used.[9] 3-Chloroacetanilide (Eastman) was purified (m.p. 73 °C)[12] by recrystallization from 50% aqueous ethanol. 3-Chloro-4-hydroxyaniline was prepared by the reduction of 3-chloro-4-hydroxynitrobenzene (Eastman) with sodium hydrosulfite in 10% aqueous sodium hydroxide, according to the method of Christiansen.[13] The crude compound was recrystallized from water in the presence of sodium bisulfite to m.p. 153 °C. Acetylation with equimolar quantities of acetic anhydride in benzene yielded 3-chloro-4-hydroxyacetanilide (m.p. 143 °C).[13] 3-Chloro-6-hydroxyaniline was prepared similarly from 3-chloro-6-hydroxynitrobenzene (Eastman). It was rapidly recrystallized from water in the presence of a small amount of sodium hydrosulfite to prevent oxidation. The gleaming white amine, stable in air, melted at 140 °C; the m.p. recorded by Mottier[14] for this compound was 138 °C. 3-Chloro-6-hydroxyacetanilide (m.p. 185 °C)[14] was obtained in good yield by acetylation of the amine with acetic anhydride in benzene.

Metabolites of 2-chloroacetanilide. 2-Chloroaniline (Eastman) was purified by distillation. The fraction distilling at 206–207 °C at atmospheric pressure was taken.[12] 2-Chloroacetanilide (Eastman) was recrystallized (m.p. 88 °C)[12] from 50% aqueous ethanol. 2-Chloro-4-hydroxyaniline was prepared by the method of Hodgson and Kershaw,[11] m.p. 160°. 2-Chloro-4-hydroxyacetanilide was prepared by acetylation of the amine with equimolar quantities of acetic anhydride and recrystallized from absolute ethanol to m.p. 124–125 °C; Hurst and Thorpe[15] report m.p. 121 °C. The infrared spectrum, however, had one strong carbonyl band at 5·9 μ, a finding indicating the presence of only one acetyl group, and a broad medium band centered at 3 μ, an observation which demonstrates the presence of a free hydroxy group. The compound was soluble in dilute aqueous sodium hydroxide, a circumstance which indicates that the hydroxy group was unsubstituted (Found: N, 7·5; Cl, 19·7; Calc. for $C_8H_8O_2NCl$: N, 7·5; Cl, 19·1%). 2-Chloro-6-hydroxyaniline was synthesized by reduction with sodium hydrosulfite of 2-chloro-6-hydroxynitrobenzene, in a manner similar to that used by Christiansen[13] for 3-chloro-4-hydroxyaniline. The intermediate nitro compound was prepared by the method of Hodgson and Moore.[16] 2-Chloro-6-hydroxyacetanilide was obtained by acetylation of the corresponding amine hydrochloride in water. The amine was suspended in water and 10 N Cl was added dropwise until the compound

dissolved. The solution was warmed to 50 °C and 1·15 Equiv. of acetic anhydride were added while the solution was stirred vigorously. Saturated aqueous sodium acetate solution, in amount sufficient to neutralize the acidity of the solution and to precipitate the product, was added immediately. The compound was washed with water and recrystallized from aqueous ethanol, m.p. 190–191 °C (Found: Cl, 19·2; Calc. for $C_8H_8O_2NCl$: Cl, 19·1%). The infrared spectrum revealed one strong carbonyl band at 6 μ and a broad medium (hydroxy) band centered at 3·2 μ; hence, acetylation did not affect the hydroxyl group.

Enzymes. β-Glucuronidase (bacterial powder, 25,000 units/g) was purchased from the Sigma Company, St. Louis, Mo. Takadiastase, a plant sulfatase, was purchased from Parke, Davis and Company, Detroit, Mich.

Methods

Care of animals and collection of urine. Male Sprague–Dawley rats (390–490 g) were kept in metabolism cages that permit the collection of urine free from feces. All animals had free access to Purina Laboratory Chow and water. The animals were kept in an air-conditioned room at a constant temperature of 22 °C. The rats were injected intraperitoneally with either 4-, or 3- or 2-[^{36}Cl]chloroacetanilide in a single dose: 100 mg per kg of body weight. The compounds were suspended in a 2 per cent methyl cellulose solution. Urine samples from animals receiving the same compound were pooled; two rats were used for 2-[^{36}Cl]chloroacetanilide and for the 4-isomer, while three rats were administered the 3-isomer. The urine was collected for a period of 72 hr after injection, in vials which were packed in dry ice; thus, the urine was frozen as soon as it was delivered into the vial. The vials were changed every 4 hr, in order that the urine was given no opportunity to thaw. Thus, bacterial or enzymatic action was prevented. The radioactivity in each 4 hr urine sample was determined by counting duplicate planchets, each plated with 0·1 ml of urine. The most active urine samples were pooled for use in quantitative determinations and qualitative identifications of metabolites, in inverse isotope dilution experiments.

General counting procedure. A carefully weighed amount (approximately 200 mg) of the postulated metabolite was added to a sample (3 or 5 ml) of urine (giving 33,800 to 78,000 counts/min) and the inactive compound was brought into equilibrium with any chemically identical radioactive material in the urine sample by refluxing for 5 hr (equilibrium was not established in some of the qualitative studies in which a milder procedure was used). The metabolite was precipitated by cooling and brought to constant specific radioactivity by successive crystallizations from several solvents. In some cases this was done following conversion to the acetyl derivative. For determination of specific activity, 0·2 ml of a solution (5 mg/ml) in 95% aqueous ethanol was plated on duplicate planchets in an almost infinitely thin layer.

To determine when the constancy of radioactivity was reached in some cases, in which several recrystallizations were made from the same solvent, activity of the recrystallizing medium was taken. The metabolite was dissolved, allowed to crystallize slowly in a centrifuge tube, left for 12 hr at 5 °C ,and then centrifuged for 5 min. The supernatant fraction was removed immediately with a sintered-glass filter stick and 0·1 ml was plated on duplicate planchets. Each sample was plated at the same temperature, thus insuring equal saturation of the solvent with the solute. When the activity of the recrystallizing medium was constant for at least two recrystallations,

the solid phase was also assumed to have constant activity; the specific activity of the crystallized metabolite was then determined.

All planchets were counted for at least 4840 counts or, if the activity was low, for a period of at least 2 hr. Background was counted for periods of 2 hr on three or more occasions during each day. All measurements were made in a Q-gas counting chamber with a thin window (Nuclear Corporation of Chicago, model D-47) and an automatic sample changer (model C-110) and a scaler (model 183) with an efficiency of 20 per cent, as calculated against a ^{60}Co standard. The amount of metabolite (expressed as percentage of radioactivity in the urine) was calculated with the aid of the formula:

$$\% \text{ metabolite} = \frac{(\text{mg non-radioactive compound added}) \times (\text{specific activity of compound recovered in counts/min per mg})}{\text{total activity of the urine sample in counts/min}} \times 100$$

Determination of the metabolites. Quantitative determinations were made of: (a) the unchanged parent compounds; (b) the N-deacetylated parent compounds; (c) the total hydroxy metabolites of each hydroxyaniline derivative, in order to determine the total amount of hydroxylation in a given position; and (d) inorganic chloride ion; in addition, qualitative studies were made to determine the presence in the urine of: (e) the unconjugated monohydroxy derivatives of the parent compounds and of the N-deacetylated compounds; and (f) the glucuronides and etheral sulfates of the monohydroxy derivatives of the parent compounds and N-deacetylated compounds. These estimations of the individual, conjugated and unconjugated, monohydroxy derivatives are considered as only qualitative since the sum of the recoveries of the individual metabolites hydroxylated in a given position did not equal the total hydroxy derivative obtained following complete acid hydrolysis. Although the same general methods were used as for the quantitative studies, it is possible that enzyme hydrolysis was incomplete and equilibrium between non-radioactive and radioactive metabolites could not always be established.

(a) *Unchanged parent compounds.* For addition to the urine samples, 2-chloroacetanilide and 4-chloroacetanilide were dissolved in acetone, and 3-chloroacetanilide was dissolved in 95% aqueous ethanol. Following refluxing, all three compounds precipitated on cooling to 5 °C. 2-Chloroacetanilide was purified by successive crystallization from 50% aqueous ethanol to radioactive constancy of the recrystallizing medium. 3-Chloroacetanilide was recrystallized successively from 50% aqueous ethanol, 50% aqueous methanol, 30% aqueous acetic acid and four times from 25% aqueous ethanol. 4-Chloroacetanilide was recrystallized from 50% aqueous ethanol and further purified by deacetylation and reacetylation as follows: the compound was was refluxed 30 min in 10% sodium hydroxide to remove the acetyl group, the reaction mixture was then cooled, made neutral with 2 N H_2SO_4 and extracted with benzene; the 4-chloroaniline was reacetylated with acetic anhydride in the extraction solvent. The solvent was distilled off and the 4-chloroacetanilide recrystallized to constant radioactivity from 50% aqueous methanol and twice from 50% aqueous ethanol.

(b) N-*Deacetylated parent compounds.* 2-Chloroaniline and 3-chloroaniline were dissolved in 95% aqueous ethanol. After refluxing with the urine samples, the solutions were cooled to 50 °C and the compounds precipitated as the acetyl derivatives in the following manner: HCl was added to form the amine hydrochloride, and acetic acid and a saturated aqueous solution of sodium acetate were added in immediate succession.

The 2-chloroacetanilide was recrystallized six times from 50% aqueous ethanol and the 3-chloroacetanilide was successively crystallized from 50%aqueous ethanol, 25% aqueous methanol, 25% aqueous acetic acid, and six times from 25% aqueous ethanol to constant activity of the recrystallizing solvent. 4-Chloroaniline was added to the urine in solution in acetone. After refluxing, the compound, which precipitated on cooling, was recrystallized from 50% aqueous ethanol and acetylated in benzene with acetic anhydride. The 4-chloroacetanilide was successively crystallized from 30% aqueous methanol and three times from 50% aqueous ethanol. For the calculations, the weights of the acetyl derivatives equivalent to the non-radioactive chloroanilines added, were used.

(c) *Total hydroxy metabolites*. The radioactive urine samples were each refluxed with 5 ml of 10 N H_2SO_4 for 1 hr to assure removal of the acetyl, sulfate and glucuronide moieties from the hydroxy derivatives. The solutions were then cooled and made neutral with 10 N NaOH. A carefully weighed amount (about 200 mg) of the monohydroxyaniline metabolite to be tested for was added in acetone and the mixtures were refluxed for 5 hr. At the end of the refluxing period the reaction mixtures were cooled and sodium sulfate was removed by filtration and twice washed with acetone to remove any occluded metabolite. In each case, acetone washings and filtrate were combined and the volume of solvent was reduced by distillation; 2-chloro-6-hydroxyaniline and 4-chloro-2-hydroxyaniline precipitated when the solutions were cooled to 5 °C. The former compound was recrystallized from 50% aqueous ethanol and then seven times from water (containing sodium hydrosulfite). The latter compound was recrystallized six times from water (in the presence of sodium hydrosulfite). To precipitate the 2-chloro-4-hydroxyaniline it was necessary to adjust the pH of the solution to 8. The product was recrystallized from water (containing sodium hydrosulfite) to constant activity of the recrystallizing medium. 3-Chloro-4-hydroxyaniline and 3-chloro-6-hydroxyaniline precipitated with the sodium sulfate. Repeated washings with water dissolved the inorganic sulfate. 3-Chloro-4-hydroxyaniline was recrystallized from 50% aqueous ethanol and then seven times from water (containing sodium hydrosulfite); 3-chloro-6-hydroxyaniline was successively crystallized from water, 50% aqueous ethanol, 50% aqueous methanol, and five times from water to constant activity of the solvent.

(d) *Inorganic chloride ion*. A sample of urine was placed in a 15 ml conical centrifuge tube and acidified with 6 N nitric acid. The urine was heated to 50 °C in a water bath and 0·1 M silver nitrate was added dropwise. Coagulation of the formed silver chloride was induced by stirring and heating. When no further precipitation occurred, the solid was spun down. The supernatant fraction was tested for unprecipitated chloride ion and was discarded when none was detected. The precipitated silver chloride was washed three times with 6 ml of water and once with 95% aqueous ethanol, dissolved in 12 N ammonium hydroxide, and filtered by means of a filter stick into a clean tube. The filter stick was thoroughly washed with 6 N ammonium hydroxide. The silver chloride was reprecipitated with nitric acid, and the procedure of washing, re-solution, and transfer to a clean tube was repeated twice; finally, transfer was made to a tared tube. The final precipitate of silver chloride was dried at 120 °C for 4 hr. The carefully weighed material was then dissolved in such an amount of 12 N ammonium hydroxide that each 0·1 ml contained 1 mg of silver chloride; 0·1 ml was plated on each of two planchets and the radioactivity was recorded. The process was repeated until constant

radioactivity of the silver chloride was reached. The percentage of radioactivity in the urine attributable to [^{36}Cl]chloride ion was determined from the formula:

$$\% \text{ chloride ion} = \frac{\text{specific activity of AgCl in counts/min per mg} \times \text{mg AgCl}}{\text{total activity of urine sample in counts/min}} \times 100$$

(e) *Unconjugated monohydroxy derivatives.* The hydroxychloroacetanilides and hydroxychloroanilines were added to the urine samples in solution in 95% aqueous ethanol. These mixtures were then refluxed for 5 hr, except for 4-chloro-2-hydroxyacetanilide and 4-chloro-2-hydroxyaniline which were stirred at room temperature for 1½ hr. Cooling of the solutions to 5 °C was sufficient to precipitate each compound. 2-Chloro-4-hydroxyacetanilide, 2-chloro-4-hydroxyaniline and 4-chloro-2-hydroxyaniline were repeatedly crystallized from water; with the aniline compounds sodium hydrosulfite was present. The other unconjugated monohydroxy derivatives were brought to constant specific activity by successive crystallizations as follows: 2-chloro-6-hydroxyacetanilide—50% aqueous acetone, 50% aqueous ethanol, 25% aqueous methanol, and twice more from 50% aqueous ethanol; 2-chloro-6-hydroxyaniline—twice from 50% aqueous ethanol and five times from water containing sodium hydrosulfite; 3-chloro-4-hydroxyacetanilide—25% aqueous ethanol, 25% aqueous acetic acid, 25% aqueous methanol, and six times from 25% aqueous ethanol; 3-chloro-4-hydroxyaniline—25% aqueous ethanol, 25% aqueous methanol, 25% aqueous acetic acid, and five times from water containing sodium hydrosulfite; 3-chloro-6-hydroxyacetanilide—50% aqueous ethanol, 50% aqueous methanol, 50% aqueous acetic acid, and again five times from 50% aqueous ethanol; 3-chloro-6-hydroxyaniline—25% aqueous ethanol and six times from water containing sodium hydrosulfite; 4-chloro-2-hydroxyacetanilide—30% aqueous methanol, 25% acetic acid, and four times from 50% aqueous ethanol.

(f) *Glucuronides and ethereal sulfates of the monohydroxy derivatives.* Each radioactive urine sample, added to a 5 ml portion of potassium phosphate buffer (pH 6·8), was incubated 24 hr at 37·5 °C with 5 ml of either 1% aqueous β-glucuronidase or 1% aqueous Takadiastase to remove the glucuronide or ethereal sulfate moieties. After incubation each mixture was filtered to remove any excess enzyme. The inverse isotope dilution technique was then applied in the same manner as for unconjugated hydroxy derivatives described in (e) above. The amount of hydroxy metabolite present in the samples after enzymatic hydrolysis was calculated with the aid of the isotope dilution formula. In each case this value was then subtracted from the amount of corresponding free hydroxy metabolite previously determined, in order to establish the presence of the metabolite freed by the enzyme.

RESULTS AND DISCUSSION

In our qualitative studies attempt was made to identify those monohydroxy metabolites which could be expected on purely chemical grounds from the fact that amino, acetylamino and chloro substituents are *ortho–para* directing, the amino group being the most powerful in this respect and the chloro group the least.

4-Chloro-2-hydroxyacetanilide and the deacetylated derivative, 4-chloro-2-hydroxyaniline were found unconjugated and also as the glucuronides and ethereal sulfates. The pattern of conjugation was different for the hydroxy metabolites of 2-chloroacetanilide and the hydroxy metabolites of 3-chloroacetanilide. Both 3-chloro-4-hydroxyacetanilide and its deacetylated derivative were found unconjugated. and also

as ethereal sulfates. 3-Chloro-4-hydroxyacetanilide, but not 3-chloro-4-hydroxyaniline, was conjugated as the glucuronide. A variation exists with 3-chloro-6-hydroxyacetanilide and its deacetylated derivative. Here both metabolites were found unconjugated and conjugated as the glucuronides, but only the acetylated compound existed as the ethereal sulfate. Neither 2-chloro-4-hydroxyaniline nor 2-chloro-6-hydroxyaniline was found unconjugated; both gave glucuronides and ethereal sulfates. On the other hand, of the corresponding acetylated metabolites, 2-chloro-4-hydroxyacetanilide was identified in all three forms, while 2-chloro-6-hydroxyacetanilide was found only as the ethereal sulfate. This last compound was the only N-acetylated hydroxy metabolite of the five isomers investigated which was not found in an unconjugated form. Each of the administered chloroacetanilides was also excreted in the urine partly as the deacetylated chloroaniline. Thus, N-deacetylation of the chloroacetanilides can occur independently from hydroxylation.

The results of the quantitative determinations of excreted metabolites are given in Table 1. In previous investigations[6] the administered compounds were found to have

TABLE 1. QUANTITATIVE RECOVERY OF RADIOACTIVITY FROM THE URINE OF RATS INJECTED WITH 4-, 3- OR 2-[^{36}Cl]CHLOROACETANILIDE

(Expressed as percentage of radioactivity in urine)

Compound	4-Chloro-acetanilide	3-Chloro-acetanilide	2-Chloro-acetanilide
Unchanged chloroacetanilides	4·63	1·34	3·18
Chloroanilines	2·53	8·26	28·28
4-Hydroxy derivatives (*para* to amino group)	—	57·32	45·10
2-Hydroxy derivatives (*ortho* to amino group)	61·96	—	—
6-Hydroxy derivatives (*ortho* to amino group)	—	26·49	8·53
Total hydroxylation (sum of lines 3, 4 and 5)	61·96	83·81	53·63
Inorganic chloride ion	1·18	1·98	0·62
Total percentage	70·30	95·39	85·71

the following decreasing order of both toxicity and antipyretic activity: 4-chloroacetanilide, 3-chloroacetanilide, 2-chloroacetanilide. For convenience in discussion, the parent compounds have been listed in Table 1 in this decreasing order. The amount of unchanged parent compound in each case was less than 5 per cent and did not follow the pattern of toxicity. The amounts of deacetylated parent compounds (i.e. 4-, 3- and 2-chloroaniline; line (2) followed a pattern: the most toxic compound, 4-chloroacetanilide, was the least deacetylated, while the least toxic compound, 2-chloroacetanilide, was deacetylated to the greatest extent. This order is contrary to the expectation that the more deacetylated chloroaniline formed, the more toxic the parent compound should be, since it is known that aniline is much more toxic than acetanilide.[17] This apparent inverse relation between the extent of deacetylation and toxicity of the substituted acetanilides may be attributable to increased solubility in body fluids and, thus, to faster elimination of the compound as a result of deacetylation. In fact, more rapid elimination occurs with 2-chloroacetanilide than with

4-chloroacetanilide. Thus, in the rat peak excretion occurred at 6 hr following intraperitoneal injection of 2-chloroacetanilide, but not until 24 hr with 4-chloroacetanilide.[18]

The total amount of hydroxylation in a given position, as determined after acid hydrolysis of the acetyl, glucuronide and ethereal sulfate derivatives, is given in lines (3), (4), (5) and (6) of Table 1. With 4-chloroacetanilide, hydroxylation cannot occur in the 4-position, since it is blocked by the chloro-sustitituent. The very small amount of inorganic chloride ion found in the urine (Table 1, line 7) shows that the block in the 4-position remains substantially intact. Initially, it was thought that the absence of *p*-hydroxylation may account for the high toxicity of 4-chloroacetanilide. For the two chloroacetanilides with the 4-position free, however, 2-chloroacetanilide exhibited less hydroxylation in the 4-position than did the more toxic 3-chloroacetanilide.

The extent of hydroxylation that occurred in the 2- and 6-positions (both of which are *ortho* to the amino group) followed the pattern of relative toxicity and antipyretic activity. 4-Chloroacetanilide exhibited the most extensive *ortho*-hydroxylation; this occurred to an extent almost two and a half times that found for 3-chloroacetanilide and more than seven times that found for 2-chloroacetanilide. Unfortunately, these experiments had to be terminated before the LD_{50} values for the 2- and 6-hydroxylated metabolites could be determined, since the results might have supported the suggestion that the toxicity of the parent compounds is related to the amount of *o*-hydroxy compound formed. The apparent relation of *o*-hydroxylation to the physiological activities of the chloroacetanilides is analogous to that found for the carcinogenic activity of certain aromatic amines. Bonser *et al.*[1] have shown a relationship between the amount of 2-aminonaphthalene that is excreted by an animal as an *o*-hydroxy metabolite, and the carcinogenic response to the parent compound. Weisburger *et al.*[2] have found that in a susceptible species, the rat, a much greater total *o*-hydroxylation of 2-acetylaminofluorene occurs than in a resistant species, the guinea pig.

The extent of total recovery of radioactivity (Table 1, line 8) indicates that metabolites other than those investigated must be present in the case of 4-chloroacetanilide and possibly in the case of the 2-isomer. Mercapturic acid formation may account in part for this low recovery. Although aromatic amines are not known to form mercapturic acids, halogen derivatives may be converted by certain mammalian species to this types of metabolite. Bray *et al.*[19] found that mercapturic acid formation is a minor metabolic pathway for 2- and 4-chloronitrobenzene, but not for 3-chloronitrobenzene. In the two former compounds, mercapturic acid apparently is formed by acetylcysteyldechlorination. On the other hand, mercapturic acids from monochlorobenzene and polychlorobenzene are products of acetylcysteyldeprotonation.[20] The small amount of free chloride ion excreted in the chloroacetanilide series (Table 1, line 7) suggests that if mercapturic acid formation takes place in these compounds it does so largely through acetylcysteyldeprotonation, rather than through acetylcysteyldechlorination. Other possible metabolites which were not tested for are polyhydroxy compounds, N-glucuronic acids, sulfamic acids and products of aromatic ring breakage.

In conclusion, no simple unifying rule can be formulated in the chloroacetanilide series which completely correlates toxicity, antipyretic activity and relative participation in the metabolic routes investigated. The only consistent pattern is that of

o-hydroxylation, which follows the same order as the relative toxicities and antipyretic activities.

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Biochemical Pharmacology, 1960, Vol. 5, pp. 39–45. Pergamon Press Ltd., Printed in Great Britain

ROLE OF PHOSPHOLIPASE A AND C IN MAST CELL DEGRANULATION INDUCED BY NON-PURIFIED *CLOSTRIDIUM WELCHII* TOXIN

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(*Received* 24 *March* 1960)

Abstract—From the observation that toxin from *Clostridium welchii* causes degranulation of mast cells it has been concluded that phospholipase C is responsible for this effect.[6,7] In the present study it has been shown that the mast cell degranulating agent is not phospholipase C but probably phospholipase A, originating from the sterile culture medium.

In some recent papers from our laboratories it has been suggested that synthetic polymer amines such as compound 48/80, "histamine liberators" isolated from *Ascaris suis* and *Cyanea capillata*, and antigen induce degranulation of rat mast cells by activating a lytic enzymatic process in the mast cell.[1–5] For reasons mentioned in these papers the enzyme was thought to be a phospholipase, related to or identical with phospholipase A.

It was observed[6,7] that mast cells were degranulated when exposed to *Clostridium welchii* toxin, and concluded that the degranulation was caused by α-toxin, the toxin assumed to be identical with phospholipase C. Some of our observations, e.g. the action of various enzyme inhibitors on mast-cell disruption, did not lend weight to the assumption that phospholipase C was the enzyme in question, but rather contradicted it.

The aim of the present study was to investigate the nature of the mast cell degranulating factor in the *Cl. welchii* toxin.

MATERIALS AND METHODS

Preparation of crude toxin

Medium for cultivation of *Cl. welchii*[8] contained pancreatic digest of casein, Na_2HPO_4, KH_2PO_4, $MgSO_4$, dextrin and thioglycollic acid. For sterilization the medium was heated to 100 °C for 20 min and to 120 °C for 30 min.

To 155 ml of this medium, the pH of which had been adjusted to 7·5, was added 0·5 ml of a *Cl. welchii* stock culture (strain CN 1491). The inoculated broth was kept at 35 °C for 16 hr and in the refrigerator for another 8 hr. Some 25 ml of this seed culture was inoculated into the vessel used for toxin production. The same medium was used, and the culture was kept at 35 °C for 16 hr. The culture was then run through a Seitz filter. The filtrate was concentrated by freeze-drying, dialysed against distilled water, then freeze-dried again. Finally it was dissolved in distilled water.

Fractionation of mast-cell degranulating factor

A sample of 3 g of IRC 50 (XE-64) was buffered with O.I.M. H_4NOH–HAc to pH 7·6. A toxin preparation (9 ml) was carefully stirred with the resin for 10 min and allowed to stand at 4 °C for 1 hr. It was then charged into a chromatogram tube of 10 mm diameter, washed with 10 ml distilled, demineralized water, then eluted with 0·1 N H_4NOH.

Fractionation of α-toxin

Cl. welchii α-toxin was prepared by ammonium sulphate fractionation and nucleic acid precipitation.[9] In order to obtain a stable preparation it was dialyzed against glycerol at 3 °C.

Phospholipase A. Prepared from bee venom.

Observations on mast cells

The technique was that described by Högberg, and Uvnäs[1] with the modification that the mesentery was incubated in a buffered solution (pH 7·0) containing $1{\cdot}6 \times 10^{-1}$ M NaCl, 3×10^{-3} M KCl, 9×10^{-4} M $CaCl_2$ (anhydrous) and 10 per cent Sörensen phosphate buffer (3×10^{-2} M Na_2HPO_4, 2 H_2O + $3{\cdot}5 \times 10^{-2}$ M KH_2PO_4).

Haemolysis

The haemolytic activity was determined on washed erythrocytes of rat. The buffered solution described in the last preceding section was used. Each test tube contained 0·4 ml of the solution to be tested, 0·4 ml of inhibitor (or buffer) and 3·2 ml of the erythrocyte suspension (about 2 per cent). The tubes were incubated at 37 °C for 3 hr. The reaction was stopped by centrifuging at 5000 rev/min for 5 min. The extinction was determined in a Hilger Biochem Colorimeter at 5500 Å, using 13 mm round cuvettes.

Manometric procedure

The reactivity of phospholipase A and C can be measured by manometric procedures.[10,11] The reaction mixture in our experiments had the following composition: 2·0 ml 4% lecithin (or phosphatidyl ethanolamine) in water; 0·3 ml M $NaHCO_3$; 0·3 ml distilled water; 0·3 ml enzyme solution, diluted in 1% albumin and 0·004 M $CaCl_2$. The flasks were filled with 100% CO_2. In experiments with antitoxin the toxin and antitoxin were pre-incubated 30 min before being put into the substrate-containing mixture.

RESULTS

Well-known effects of phospholipase C are the hydrolysis of lecithin and the (direct) lysis of erythrocytes. From Fig. 1 and 2 it is evident that our *Cl. welchii* toxin has both

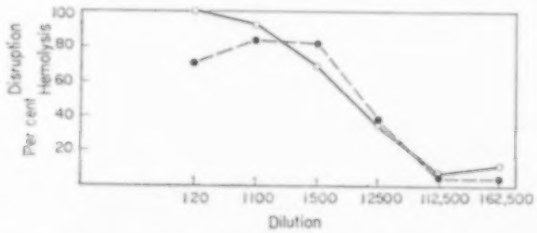

FIG. 1. Haemolysis ● —— ● —— ● and mast cell degranulation ○——○——○ caused by crude *Clostridium welchii* toxin.

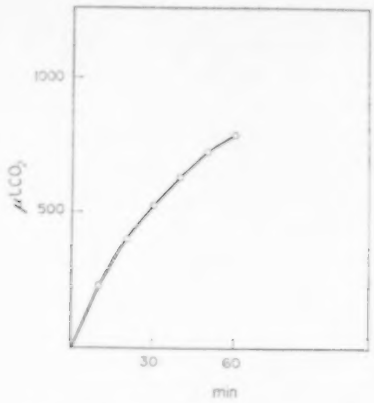

FIG. 2. Hydrolysis of lecithin caused by crude *Clostridium welchii* toxin.

of these properties and in addition degranulates mast cells. The apparent correlation between these actions is consonant with the idea that they might be due to one and the same factor, i.e. phospholipase C. However, several arguments can be presented against this conclusion:

(1) Antitoxin against *Cl. welchii* inhibited the haemolytic action of the toxin but not its *mast cell degranulating* action (Fig. 3).

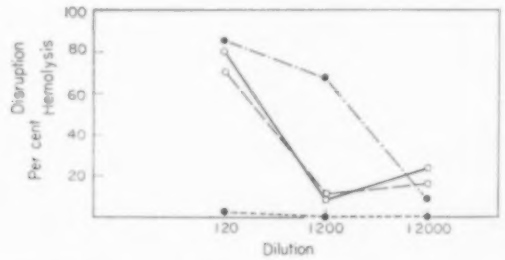

FIG. 3. Inhibition by *Clostridium welchii* antitoxin of *haemolysis* caused by crude toxin. ● - . - . - ● - . - . - ● without antitoxin; ● . . . . . ● . . . . . ● with antitoxin. Note the absence of inhibition of mast-cell degranulation, ○ —— ○ —— ○ without antitoxin; ○ - - - ○ - - - ○ with antitoxin.

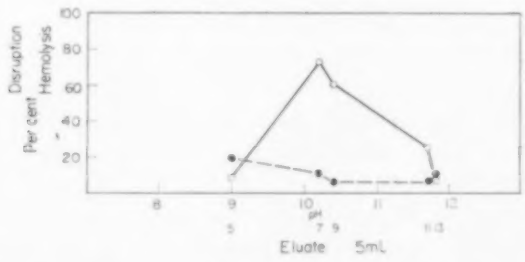

FIG. 4. Separation of mast-cell degranulating factor ○——○——○ from haemolytic factor ● - - - ● - - - ● in crude *Clostridium welchii* toxin. Small figures refer to number of the elutate. Dilution 1:40.

(2) Using Amberlite IRC 50 (XE-64), the mast cell degranulation factor in the toxin preparation was separable from the haemolytic one (Fig. 4). This latter factor was not recovered from the resin. Possibly it was denatured.[12]

(3) With salt fractionation and nucleic acid precipitation it was possible to obtain fractions with high haemolytic and lecithin splitting activities. These fractions did not hydrolyse phosphatidyl ethanolamine, nor did they degranulate mast cells. The hydrolytic activity was blocked by *Cl. welchii* antitoxin (Figs. 5a, b and c).

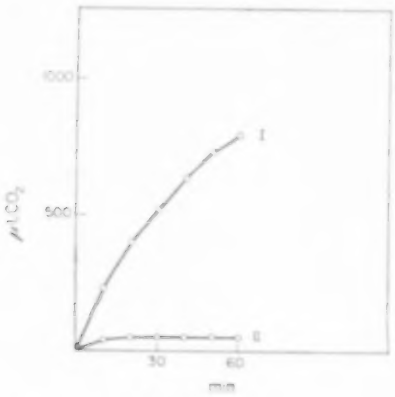

Fig. 5(a). Hydrolysis of lecithin produced by partly purified *Clostridium welchii* toxin (I). Note the absence of phosphatidyl ethanolamine hydrolysis (II).

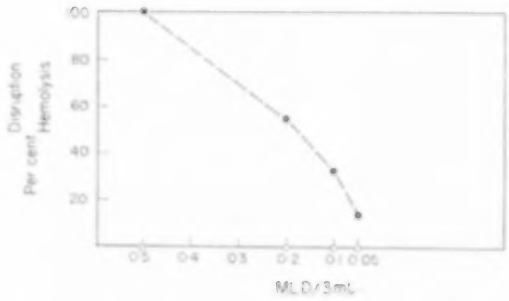

Fig. 5(b). Haemolytic action of partly purified *Clostridium welchii* toxin ●----●----●. Note the absence of mast cell degranulation ○——○——○.

(4) It was observed that even the sterile culture medium contained a mast cell degranulating factor. There was no increase of the degranulating activity after the growth of *Cl. welchii* (Fig. 6).

The above observations militate against the supposition that the mast cell degranulation is due to phospholipase C in the toxin. It is more likely to be due to the presence of phospholipase A in the culture medium, since:

(a) Ox pancreas, an organ rich in phospholipase A, was one of the raw materials for the culture medium. It is true that the broth was sterilized by heating to 120 °C,

but phospholipase A is known to be unusually heat resistant. In control experiments extracts of ox pancreas heated to 120 °C still showed mast-cell disrupting activity (Fig. 7).

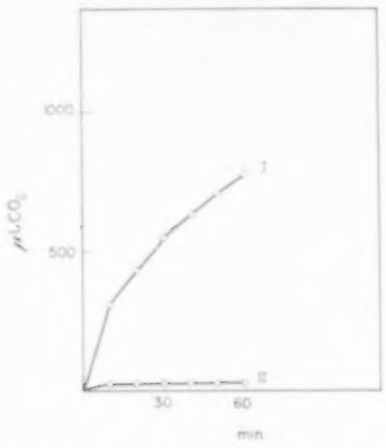

FIG. 5(c). Inhibition of lecithin hydrolysing action of *Clostridium welchii* toxin by *Clostridium welchii* antitoxin. I: Without antitoxin. II: With antitoxin.

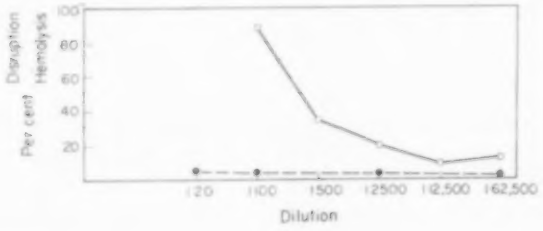

FIG. 6. Haemolysis ●- - -●- - -● and mast-cell degranulation ○——○——○ caused by sterile culture medium.

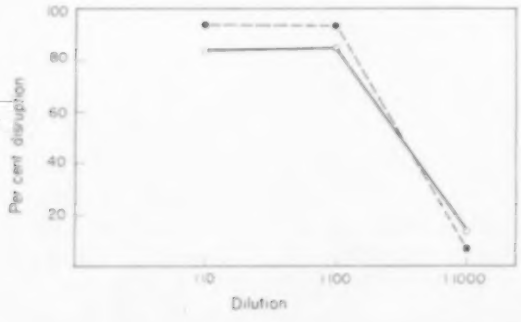

FIG. 7. Heat resistance of mast-cell degranulating factor in extract of ox pancreas. ○——○——○ untreated medium; ●- - -●- - -● sterilized medium.

(b) Phospholipase A acts on e.g. phosphatidyl ethanolamine; phospholipase C does not. With the Warburg technique it was found that the culture medium was able to split both lecithin and phosphatidyl ethanolamine (Fig. 8).

(c) Phospholipase A is able to lyse red blood corpuscles (e.g. from the rat) only in the presence of lecithin. In our experiments the sterile culture medium required lecithin for its haemolytic action. This is in contrast to the direct haemolytic activity of the toxin preparation.

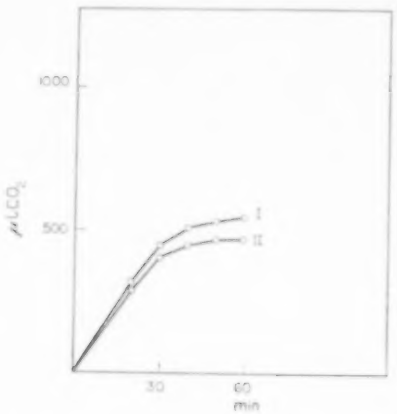

FIG. 8. Hydrolysis of lecithin (I) and phosphatidyl ethanolamine (II) produced by sterile culture medium.

DISCUSSION

Some properties of phospholipase A and C, including the influence of *Cl. welchii* antitoxin, are listed in the Table 1. The data indicate that the mast-cell degranulating

TABLE 1. SOME PROPERTIES OF PHOSPHOLIPASE A AND PHOSPHOLIPASE C

	Hydrolysis of lecithin	Hydrolysis of phosphatidyl ethanolamine	Direct haemolysis	Indirect haemolysis	Mast-cell degranulation	Inhibition by *Cl. welchii* antitoxin
Phospholipase A	+	+	0	+	+	0
Phospholipase C	+	0	+	−		+

factor in our toxin preparation was not phospholipase C: The mast cell degranulation caused by the toxin was not inhibited by *Cl. welchii* antitoxin. Fractions causing haemolysis and hydrolysis of lecithin *in vitro* did not degranulate mast cells and did not attack phosphatidyl ethanolamine. The haemolytic and lecithin-splitting actions were blocked by *Cl. welchii* antitoxin. Evidently it is possible to separate a phospholipase C fraction showing no mast-cell degranulating ability.

In fact the mast-cell degranulating factor does not even seem to be formed by the bacteria. It already occurs in the sterile culture medium, and the observations are

consistent with the assumption that it is phospholipase A: It contains a principle that splits phosphatidyl ethanolamine, and it requires lecithin for its haemolytic action.

Högberg and Uvnäs[1] suggested that the degranulation of mast cells induced by compound 48/80, agents extracted from *Ascaris suis* and *Cyanea capillata*, and antigen was due to an enzymatic process localized in the mast cell, involving the activation of a lytic enzyme attacking the phospholipids of the membranes. The influence of inhibitors suggested that the enzyme is related to phospholipase A but not to phospholipase C. The observation that the mast-cell degranulating factor in the crude *Cl. welchii* toxin is not phospholipase C but probably phospholipase A tends, in our opinion, to support our theory.

Acknowledgements—Sterile culture medium and crude toxin filtrate were supplied by Statens Bakteriologiska Laboratorium, Solna. This work was supported by a grant from Statens Medicinska Forskningsråd, Försvarsmedicinska nämnden.

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CHANGES IN THE LIPID COMPOSITION OF TUMOUR AND HEPATIC TISSUES OF RATS BEARING GUÉRIN CARCINOMA FOLLOWING TREATMENT WITH THE NITROGEN MUSTARD DEGRANOL*

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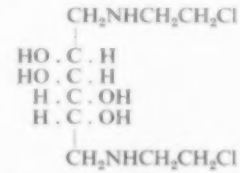
Abstract—Degranol had no effect on the total lipid content of the whole tumour if referred to dry tissue. In the florid tumour part it reduced the glycerides and increased the phospholipids, while in the necrotic part it increased the former and reduced the latter in amount. In the hepatic tissues it caused a fall of the glycerides and produced a smaller rise of the phospholipids. It also caused a rise in the fatty acids in all three types of tissue, reaching peaks at 96 hr after injection. No changes were observed in total cholesterol after giving the drug. At 24 and 48 hr following treatment, the polyunsaturated spots in the fatty acid components of the phospholipids were found to have decreased. The fatty acid components of the glycerides in the nectrotic tumour tissues contained stearin, palmitin, linoleic acid, and in addition, polyunsaturated fatty acids. The spot of the latter was found to have disappeared at 24 hr after Degranol. At 72 hr it reappeared and by that time it was also seen in the florid tumour tissues.

MALIGNANT tumours show excessive accumulation of fat which is not seen in normal tissue.[1] Their necrotic parts reveal characteristic changes in total lipid composition: glycerides decrease in amount while steroids and fatty acids increase.[2] In the view of some investigators these alterations characterize every type of tumour,[3] in that of others they are primary in certain tumour types but secondary in the rest.[4] Cytotoxic agents materially influence the histology of transplanatable rat tumours.[5]

The aim of the present work was to study the effect of Degranol (BCM) on the lipid composition of tumours; more precisely, of the tissues of the three different zones distinguishable in every tumour, namely the florid, the necrotic, and the intermediate zone. The study was at the same time extended to the hepatic tissues of the tumour bearing animal.

* Degranol is the nitrogen mustard derivative of D-mannitol, and its biological and chemical properties have been described by Keller and Németh.[5a]



Accordingly, we determined the amount of total lipid extracted from the tissues; the ratio in it of the acid-soluble to the acid-insoluble lipids; the free fatty acid content of the acid-soluble fraction; and the amount of the total, the free and the esterified cholesterol, respectively. In addition, both the composition and amounts of the fatty acids released on saponification of the glycerides and phospholipids were determined.

METHODS

Wistar rats, 3 months old and all fed the same diet throughout the experiments, were used. Subcutaneous implants of bits of Guérin tumour, the size of a pea, were made in the left interscapular region, and when the tumours reached $n = 4$ size[6] each rat was given a 50 mg/kg intravenous dose of Degranol. Nine animals at a time were killed at 24, 48, 72, 96, 120, 148 and 220 hr after injection, and their tumours and livers were pooled in sets of three for further work. The tumours were cleaned from their connective-tissue sheath and cooled to from 0° to +3 °C, after which they were divided into three parts corresponding to the macroscopically distinguishable florid, necrotic and intermediate zones.

Wet weights of the separate tumour parts were established gravimetrically, and the liver was weighed. Water was removed from the tissues by lyophylization according to the method of Hack.[7] Water content was computed from difference in weight. Total lipid was extracted with hot petroleum ether at a temperature not exceeding 80 °C, and the extract was evaporated to dryness. From the residue the glycerides, the free fatty acids, and the cholesterol were dissolved in acetone, leaving over the acetone-insoluble phospholipids. All these lipid constitutents were determined gravimetrically.

Free fatty acid content was determined with Jáky's[8] micromethod. For the purposes of evaluation a series of known fatty acid contents was prepared from 50 μg upwards. Evaluation was done by colorimetry.

Total and esterified cholesterol was determined by the Liebermann–Burchardt reaction, and free cholesterol was computed from the difference between the two.[9]

Glycerides and phospholipids were saponified in the warm in a stream of N_2. The fatty acid components were separated by paper chromatography, in a hydrophobic phase, using the paraffin impregnation method of Spiteri and Michales,[11] slightly modified. In some preliminary experiments Kaufmann's[10] petroleum impregnation procedure had been tried, but was discarded because it involved substantial loss of time and material to prepare a fraction that would distil through at temperatures between 190 and 220 °C from the commercial petroleum we had to use for the lack of "Undekan" or some similar preparation, and because it had proved very difficult to attain and maintain in the strips the optimal petroleum concentration of from 180 to 200 mg/g. Our modification of the Spiteri–Michales method was to dissolve the paraffin 15% benzene instead of 10% ether, because the form evaporates more slowly: which is an advantage when great many strips have to be impregnated. Kaufmann's[12] solvent system (75% acetic acid and 25% acetonitrile) was used in the preliminary exploratory experiments, but on paper impregnated with paraffin it yielded more than usually high R_f values and imperfectly separated spots. According to Kaufmann[13] the separation of fatty acids is brought about partly by the action of the more powerful acetic acid which represses the tendency of fatty acids to dissociate at different rates. This involves several days before adequate separation develops. Kaufmann[12] shortened separation time by including acetonitrile in the solvent system without interfering with

the part played by the acetic acid. His system is ideal when petroleum is used for impregnation, but requires modification for the use of paraffin. The solvent system used by us contained 75% acetic acid, 5% water and 20% acetonitrile, whereby we increased the dissociative capacity of the acetic acid and decreased the polarity of the acetonitrile. With its use adequate separation was achieved in 24 hr. Chromatography was carried out by ascending flow at between 16 and 24 °C on Schleicher–Schull no. 2043/b paper of 30 mm width and 300 mm length. This permitted adequate separation of from 300 to 400 μg of substance. The finished chromatograms were dried and the spots developed in copper acetate and rubeanic acid. Model fatty acids were used to identify stearin, palmitin, oil and linol. The alkaline potassium permanganate solution of Kaufmann[14] was employed in demonstrating the presence of unsaturated fatty acids, but in twice as high a dilution as he recommended. Lower concentrations sufficed to oxidize these acids, and the pale pink colour of the permanganate in no way hindered the clear distinction of spots. Qualitative determination of the chromatographically separated fatty acid spots was followed by quantitative estimation. A Juan-type photodensitometer was used, which in addition to the extinction curve draws its integral, and thus permits direct reading of the percentage of spots on the individual strips. A similar method of evaluation has been described by Seher.[15]

RESULTS

The average water content of the tissues was 75% in the liver, and 85% in the florid and necrotic tumour parts, respectively. Treatment with Degranol had no effect on it in the hepatic and the necrotic tumour tissues, but decreased it to 80 per cent in those of the florid tumour part at 96 hr after the drug. According to the data in the literature the water content is generally higher in tumours than in normal tissues.[16]

The total lipid content of the tumours of the untreated controls ranged between 8·1 and 8·9 per cent and remained largely within these limits after treatment with Degranol. For hepatic tissues the figures varied from 11 to 15 per cent in the controls, but after Degranol decreased to from 8 to 9 per cent between 24 and 148 hr, to return to the 10 to 15 per cent level after 180 hr. Lustig[17] found that the total lipid content of the normal human lung, referred to dry matter, was 8·7 per cent, and that of the tumour-bearing lung 11·26 per cent. According to Fujiwara *et al.*,[18] the total lipid amounts to 9·1 per cent of the dry matter in the tissues of the normal rat liver and 8·2 per cent of the hepatoma-bearing animal.

In the controls, the acetone-soluble lipid fraction of the whole tumour, referred to dry weight, varied from 30 to 50 mg, and the insoluble fraction from 30 to 60 mg. Treatment with Degranol had no effect on either of these levels. The acetone-soluble lipid fraction of the hepatic tissues of the untreated animals ranged between 55 and 80 mg, with a tendency to decline 24 hr after the drug. By 120 hr it sank to levels from 5 to 10 mg, thereafter to rise again but not above 80 mg. The acetone-insoluble lipid fraction of the control livers was between 30 and 60 mg, and remained unaffected by the treatment.

As regards the total lipid content of the florid and the necrotic tumour tissues, the data in the literature are at variance. Christol and Monier[19] found the former was 2·6 per cent and the latter only 1·7 per cent of the dry weight. Bullock and Cramer,[20] on the other hand, showed that in Walker carcinoma the lipid content of the necrotic tumour tissues exceed that of the florid tissues. Boyd and McEwen,[21] however, found

for the Walker carcinoma 256 no difference in the total lipid content of the florid and the necrotic tissues.

The lipid content of the acetone-soluble fraction in the florid tumour parts varied from 35 to 85 mg in the untreated controls, but in the treated animals it gradually decreased to fall to between 10 and 25 mg by 220 hr. The acetone-soluble lipid content in the necrotic parts rose from 8–25 mg in the controls to 75–90 mg in the treated animals.

The acetone-insoluble lipid fraction of the florid tumour tissues was 30—60 mg in the controls and attained between 68 and 80 mg in the treated animals. The same fraction of the necrotic tissue was 25—55 mg in the controls, and gradually decreased in the treated rats, sinking by 220 hr to levels of from 8 to 30 mg.

Table 1 shows that, expressed in μg/mg, the fatty acid content of the florid tumour parts was 80 to 90 μg in the untreated animals, and that between 96 and 120 hr after

TABLE 1. CHANGES IN FREE FATTY ACID CONTENT FOLLOWING TREATMENT WITH DEGRANOL

(μg of free fatty acid per mg of acetone-soluble fat)

Number of hours after Degranol		Florid tumour part	Necrotic tumour part	Liver
Untreated controls	1	90	300	50
	2	80	200	50
	3	80	200	50
Untreated controls	1	80	100	50
	2	80	150	50
	3	80	150	50
Untreated controls	1	80	100	50
	2	80	100	50
	3	—	—	—
24	1	50	300	80
	2	50	300	80
	3	80	300	80
48	1	50	90	80
	2	50	90	80
	3	70	90	80
72	1	80	120	100
	2	100	120	100
	3	100	120	100
96	1	200	250	200
	2	250	250	200
	3	250	250	200
120	1	200	280	—
	2	250	280	50
	3	250	280	200
148	1	200	250	200
	2	—	250	200
	3	200	250	200
220	1	90	90	50
	2	50	90	50
	3	90	90	50

treatment it was 250 μg, but fell to 50 μg by 220 hr. That of the necrotic tumour parts varied between 100 and 300 μg in the controls; in the treated rats it consistently reached a peak at 24 hr, fell to from 90 to 120 μg between 48 and 72 hr, and climbed to a second peak of from 250 to 280 μg between 96 and 120 hr with a terminal drop to 90 μg. The free fatty acid content of the hepatic tissues was 80 μg in the untreated rats; in the treated ones a peak of 200 μg at 96 hr was followed by a rather rapid decline to 50 μg by 220 hr.

As regards cholesterol, the abundant data in the literature agree in that the total amount of this constituent is significantly greater in tumours than in normal tissues,[22] and in necrotic than in florid tumour parts. Lang (cited by Haven and Bloor[22]) found that a decrease in tumour-cell activity meant an increase in cholesterol content.

The values for total cholesterol were found to vary very widely. Referred to grammes of dry tissue, they ranged between 15,000 and 20,000 μg for the florid, between 30,000 and 40,000 μg for the necrotic tumour parts, and between 5000 and 10,000 μg for the tissues of the liver. Degranol had no effect on either of them.

Of the tumour lipids, mainly the phospholipids have been subjected to more minute analyses by earlier investigators, and generally for changes in amount and the degree of unsaturation only; these changes were established by determinations of iodine numbers. Haven and associates[13] found that the ratio of the saturated to the unsaturated fatty acids of the tumour phospholipids was 30 : 70. On evidence furnished by the same authors, in animals fed the same diet the degree of unsaturation of the phospholipids in the tumours remains unchanged indpendently of the location of the tumour.[24] Kellner and Lustig[2] showed that by feeding diets rich in oil it was possible to increase the iodine number of the total fatty acids of transplanted mouse tumour. Haven found no essential difference in iodine numbers between tumour and muscle tissues: with Walker carcinoma the iodine numbers rose from 85 on a fat-poor diet, to 95 on coco-nut oil, and to 106 on cod liver oil diet, in tumour and muscle alike. This author also showed that by force-feeding of a diet high in fat it was possible to increase the amount of the total lipids of the tumour, without, however, obtaining a corresponding increase in tumour size.

Fig. 1 illustrates the fatty acid composition of the phospholipids in the florid and necrotic tumour parts and the hepatic tissues of rats bearing Guérin carcinoma. Fig. 2 shows the changes in the composition observed at 24 and 48 hr following treatment with Degranol. The components are stearin, palmitin and oil, linol, and polyunsaturated fatty acid. The hepatic tissues of the untreated animals show an additional spot of the last-mentioned constituent. At 24 and 48 hr after Degranol a marked decrease in the amount of the polyunsaturated fatty acids was observed in the florid and the necrotic tumour tissues, but at 72 hr they were present in these tissues in higher concentrations than in those of the untreated controls. From the hepatic tissues the spot due to the least saturated fatty acid disappeared at 24 hr, but reappeared at 72 hr after treatment.

Figs. 3 and 4 show the fatty acid composition of the glycerides in the florid and necrotic tumour parts and in the hepatic tissues in untreated animals, respectively, in animals at 72 hr after Degranol treatment. The components are stearin, palmitin and oil, linol, and polyunsaturated fatty acid. The necrotic tumour tissues reveal one additional spot of polyunsaturated fatty acid. At 24 and 48 hr following treatment a marked decrease in the amount of the polyunsaturated fatty acids was noted in the

FIG. 1. Fatty acid composition of the phospholipids in tumour and liver in tumour-bearing rats.

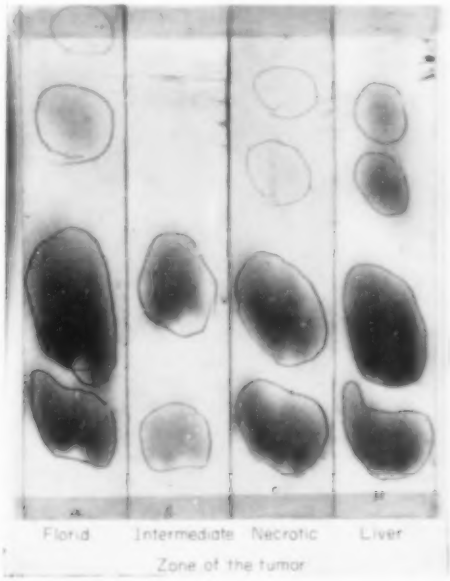


FIG. 2. Changes in the fatty acid composition of tumour and liver phospholipids 24 hr after Degranol treatment.

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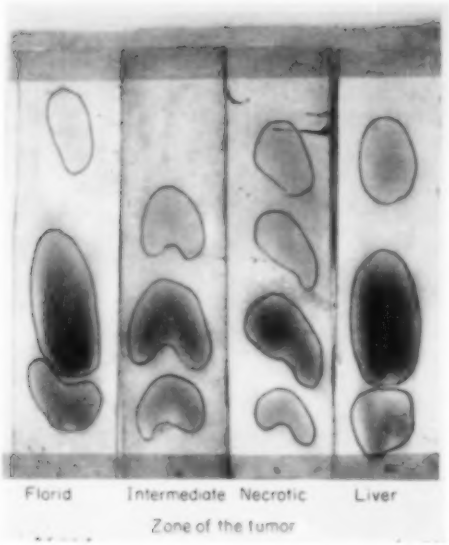

FIG. 3. Fatty acid composition of tumour and liver glycerides in tumour-bearing rats.

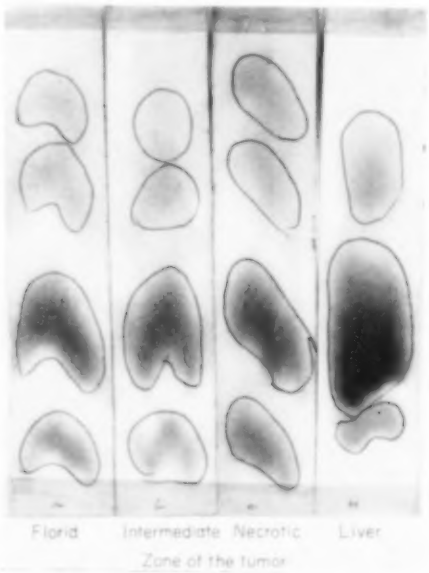

FIG. 4. Changes in the fatty acid composition of tumour and liver glycerides 72 hr after Degranol treatment.

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necrotic tumour tissues, but at 72 hr the polyunsaturated fatty acid spot was again present in the florid and necrotic tissues alike.

DISCUSSION

Degranol has been found to have no effect on the lipid content of the whole tumour, but to give rise to characteristic changes in that of each the florid and necrotic parts separated on gross inspection. Of course, in Guérin tumours, where necrotic parts sporadically infiltrate florid parts, complete separation is extremely difficult, if at all possible; this means, the values for controls and treated animals alike will be widely scattered and will render the results problematical.

The presence of fatty acid in necrotic tumour tissues was also observed by Kellner and Lustig.[2] The increase in its amount following treatment with Degranol is presumably due to a change in the activity of some kind of lipase.

The changes in the fatty acid composition of the phospholipids, which we observed to follow treatment with Degranol, undoubtedly appear to be the results of the drug's action. Support is afforded for this view by the finding that unsaturated fatty acid components decrease in amount as the morphological changes increase in severity. One of the polyunsaturated spots proved to be lineolic acid. This is an essential fatty acid, which has been shown by Deuel *et al.*[25] to be an indispensable requisite for the action of the growth hormone. The presence of polyunsaturated fatty acids in tumours, particularly in the phospholipid fraction, has been established unequivocally. The fact that a toxic dose of Degranol reduces the amount of these particular fatty acids, permits the conclusion that the drug is not without effect on tumour metabolism.

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THE CYTOCIDAL ACTION OF MITOTIC POISONS ON LYMPHOCYTES *IN VITRO*

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(*Received* 10 *May* 1960)

Abstract—Rat lymph nodes were maintained *in vitro* by an organ culture technique. The lymphocytocidal effects of the following substances were investigated: nitrogen mustard, acriflavine, quinone, hydroquinone, rotenone, cortisone, colchicine, podophyllotoxin, chelidonine, arsenite, stilboestrol, selenite, diphenylhydantoin, cyanide. This list includes all the substances known to kill lymphocytes *in vitro* at 10^{-4}M and most of the mitotic poisons shown (by others) to act on fibroblasts *in vitro* at 10^{-5}M. For each substance the LC at 50–48 hr (concentration which kills 50 per cent of the lymphocytes in 48 hr) was determined from the dose–effect curve.

In the cases of nitrogen mustard, acriflavine, rotenone, colchicine, arsenite and stilboestrol, the LC_{50} for lymphocytes was found to be about the same as the concentration required for mitotic poisoning of fibroblasts. But the mitotic poisons quinone–hydroquinone and chelidonine were not particularly poisonous to lymphocytes, and the specific lymphocyte poisons diphenylhydantoin and selenite are not mitotic poisons. The results do not encourage any simple idea of a single biochemical lesion common to lymphocyte and mitotic poisoning. Such correlation as exists may simply reflect the fact that mitosis is the cell process and the lymphocyte the cell type most vulnerable to adverse influences.

Rotenone is by far the most potent lymphocyte poison so far discovered; in 10^{-9}M concentration it kills at least one in every five cells.

INTRODUCTION

A VERY wide variety of chemical substances,[1] including distilled water,[2] have now been shown to inhibit or derange the process of cell division. Moreover, mitosis can be affected in several different ways. The term "mitotic poison" has thus come to be so broadly and variously used as to lose much of its former significance. Recent attempts to redefine the term in a more meaningful way have only revealed considerable differences of opinion.[1] So it remains the first duty of an author to explain what he means by "mitotic poisoning".

The mitotic poisons considered in this paper are those powerful poisons which specifically either prevent or arrest mitosis in a wide variety of animal cells. More precisely, they all inhibit mitosis in fibroblast cultures *in vitro* at concentrations of 10^{-5} M or less (Table 1, column E), with no other observable effect on the cells (except that a few cause chromosome breakage). Most of them have been shown to inhibit the mitosis of sea urchin eggs at similarly low concentrations (Table 1, column D). The various metabolic inhibitors of respiration, glycolysis, phosphorylation, etc., are excluded from consideration for they only act at concentrations of about 10^{-4} M or more and they affect other cell processes as well. The actions of the powerful specific mitotic poisons cannot yet be explained in chemical terms.

TABLE 1

(A1 = chromosome poisons, A2 = spindle poisons, A3 = not mitotic poisons. Literature references in brackets. In some cases the figures have been calculated from data given in the publications cited. Column C gives the minimum dose reported to cause obvious pyknosis of lymphocytes in lymph nodes or thymus within 12 hr, as seen in histological sections. Column E gives, for the chromosome poisons—the minimum concentration reported to cause a marked (usually 50 per cent) reduction in the mitotic index, for the spindle poisons—the minimum concentration reported to cause an obvious accumulation of arrested metaphases.

	B Lethal dose (rat) (mg/kg) (s.c. = subcutaneous, i.v. = intravenous, i.p. = intraperitoneal)			C Dose to kill lymphocytes in the lymphoid tissues (rat or mouse) (mg/kg)		D Concentration for mitotic poisoning of sea-urchin eggs		E Concentration for mitotic poisoning of fibroblasts *in vitro*		F LC at 50–48 hr for lymphocytes *in vitro*
A1										
Nitrogen Mustard	1·1	i.v.	(10)	1·0	(24)	10^{-3}M	(31)	10^{-5} M	(37)	$3{\cdot}7 \times 10^{-6}$ M
	1·9	s.c.	(10)							
Acriflavine	33·0	s.c.	(11)	25·0	(25)			$2{\cdot}5 \times 10^{-6}$ M	(38)	10^{-5} M
								10^{-5} M	(37, 39)	
Quinone	25·0	i.v.	(12)			7×10^{-6} M	(32)	10^{-9} M	(37)	$1{\cdot}9 \times 10^{-4}$ M
Hydroquinone	115·0	i.v.	(12)	125·0	(3)	5×10^{-6} M	(32)	6×10^{-9} M	(40)	$2{\cdot}1 \times 10^{-4}$ M
Rotenone	5·0	i.p.	(13)	10·0	(26)	5×10^{-9} M	(33)	$2{\cdot}5 \times 10^{-8}$ M	(41)	$2{\cdot}7 \times 10^{-9}$ M
	6·0	i.v.	(14)							
Cortisone				12·5	(27)	3×10^{-4} M	(34)	5×10^{-7} M	(42)	$3{\cdot}5 \times 10^{-5}$ M
A2										
Colchicine	1·7	i.v.	(15)	2·0	(28)	10^{-5} M	(35)	$2{\cdot}5 \times 10^{-8}$ M	(39)	$2{\cdot}3 \times 10^{-7}$ M
	4·0	s.c.	(16)	1·3	(29)			5×10^{-9} M	(38)	
Podophyllotoxin	8·0	s.c.	(17)	8·0	(17)	$1{\cdot}5 \times 10^{-6}$ M	(36)	10^{-5} M	(43)	10^{-7} M
Chelidonine	300·0	s.c.	(18)					$2{\cdot}5 \times 10^{-6}$ M	(44)	
Na arsenite	10·0	i.p.	(19)	2·3	(30)			$3{\cdot}4 \times 10^{-6}$ M	(45)	$2{\cdot}2 \times 10^{-6}$ M
Stilboestrol	500·0	s.c.	(20)			2×10^{-6} M	(32)	$1{\cdot}5 \times 10^{-5}$ M	(46)	$1{\cdot}2 \times 10^{-5}$ M
								4×10^{-5} M	(44)	
A3										
Na selenite	3·0	i.v.	(21)	5·0	(3)			10^{-3} M	(47)	$4{\cdot}6 \times 10^{-6}$ M
	7·5	i.p.	(19)							
Diphenylhydantoin	280·0	i.p.	(22)					$>10^{-4}$ M	(48)	$6{\cdot}3 \times 10^{-5}$ M
K cyanide	2·5	i.v.	(23)					5×10^{-5} M	(47)	$1{\cdot}6 \times 10^{-4}$ M

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Following Dustin[3], these nitotic poisons can be divided into two groups, "chromosome poisons" and "spindle poisons". The chromosome poisons, such as acriflavine and nitrogen mustard, affect the cells before division, preventing the onset of mitosis, and in many cases breaking the chromosomes as well. It seems unlikely, however, that chromosome breakage is the cause of the mitotic inhibition; recent work suggests that these are separate and unrelated effects.[4] The spindle poisons, such as colchicine and arsenic, affect cells during division, destroying the spindle and arresting the cells in metaphase. The chromosome damage in the one case or the spindle damage in the other may be so great that the cells ultimately die.

The division of mitotic poisons into these two groups has often been criticized because some poisons can exhibit both actions, depending on the concentration. But even so it seems legitimate to maintain a distinction between the two types of mitotic poisoning and the belief that this reflects two different points of chemical attack on the mitotic process. In Table 1 (column A) the mitotic poisons have been allocated to one or other of these two groups (A1, A2) on the basis of the action which they exhibit at lowest concentration.

So far, it has been implied that these poisons affect only cells in division or about to divide, but this is not the whole truth. Many of them have an additional and totally different action. Injected into animals in antimitotic doses they selectively kill the lymphocytes in all the lymphoid tissues. The cells in question are nearly all small lymphocytes which never divide (except in the thymus cortex in some species), so the question of mitotic poisoning does not arise. Small doses of ionizing radiation also inhibit mitosis in general and kill small lymphocytes in particular.

The purpose of the present work was to explore more fully this apparent parallelism between mitotic inhibition and lymphocyte killing. Using rat lymph nodes maintained *in vitro*, the various mitotic poisons were added to the culture medium and in each case the concentration range required to kill the lymphocytes was accurately determined. The concentrations needed to kill lymphocytes *in vitro* were then compared with the concentrations which have been shown to inhibit mitosis in fibroblast cultures *in vitro*. Results obtained with sodium selenite and diphenylhydantoin are also included, for comparative purposes. These two substances are specifically toxic to lymphocytes but they are not mitotic poisons, (as here defined) and they are put in column A3 in Table 1. Also included in A3 is cyanide as an example of a non-specific poison.

Investigations of this sort can be carried out only *in vitro*, for only so can a direct effect of the poison be demonstrated and the concentration applied be exactly known. In the whole animal the concentration of poison reaching the cells and its length of stay are unknown and in the particular case of lymphocytes these cells may also be killed indirectly by the pituitary-adrenal stress reaction. For these reasons, results obtained by injecting mitotic poisons into animals (Table 1, column C), though suggestive, are unacceptable for quantitative consideration.

METHODS

Suspensions of lymphocytes do not survive satisfactorily *in vitro* and it has been found better to use lymph nodes from small animals (rats) maintained *in vitro* by an organ culture technique.[5] In these lymph-node cultures the lymphocytes remain alive and healthy for about a week,[5] though there is no production of new cells by mitosis of precursors. More than 99 per cent of the lymphocytes are mature small lymphocytes.[6]

This survival time is ample for the study of chemical toxicity which was done by counting the percentage of dead (pyknotic) lymphocytes at time intervals (5–48 hr) after addition of the poison.

This method has already been used to study the cytocidal action of X-rays,[7] cortisone[8] and barbiturates[9] on lymphocytes *in vitro*. In the present work the method previously described in this *Journal*[9] was followed exactly, using the lumbar and sacral lymph nodes of 4-week-old rats. It only remains to describe how the poisons were made up and added.

The nitrogen mustard used was Mustine Hydrochloride BPC (Boots Pure Drug Co.). Podophyllotoxin and Chelidonine were obtained from L. Light and Co. Sodium-5 : 5-diphenylhydantoinate ("epanutin") was obtained from Parke, Davis and Co. Cortisone (free alcohol) and rotenone (material 51R 3753) were obtained from Merck and Co. The rotenone was about 99 per cent pure; most commercial samples are very impure. All the other substances were obtained from British Drug Houses.

"Initial" solutions were made up not more than an hour before addition to the cultures, at a concentration of 10^{-1} M for the weak poisons and 10^{-2} or 10^{-3} M for the stronger ones. Colchicine, acriflavine, sodium arsenite, sodium selenite and KCN were made up in 10 ml volumes of distilled water and the solutions sterilized by suction through a No. 5 porosity sintered glass filter. Nitrogen mustard was dissolved from a sterile ampoule in sterile distilled water and added to the cultures within 5 min. In the case of all the other substances, the required weight for 10 ml of solution was placed in a sterile graduated test tube and dissolved in 4 ml of ethanol, after which the volume was made up to 10 ml with sterile distilled water. The alcohol sterilized the solution and no filtration was necessary.

For the highest concentrations required in the culture medium the initial solution was added directly in the amount of 0·05 ml or 0·015 ml per 5 ml of medium. For lower concentrations the initial solution was first diluted 1 in 10, 1 in 100 or 1 in 1000 with sterile distilled water and then added in the amount of 0·05 or 0·015 ml per 5 ml of medium. The final concentration of ethanol in the medium never exceeded 0·4 per cent and control experiments[9] had shown that 10^{-1} M ethanol (0·46 per cent) was quite harmless.

In some cases the upper part of the effective concentration range could not be covered completely because of difficulty in solubility (cortisone, chelidonine) or cytological fixation effects (quinone, hydroquinone).

Some of the figures for cortisone,[8] colchicine[9] and cyanide[9] have been published previously in other contexts.

RESULTS

Fig. 1 shows that after addition of colchicine or rotenone, the percentage of dead lymphocytes in the culture (the "pyknotic count") increases more or less linearly with time for the first 48 hr. It has been assumed that this linear relationship also holds good for the other poisons, and 48 hr was chosen as a convenient (but arbitrary) end-point at which to measure the effect. The effects of different concentrations and different poisons were thereby compared. The results are given in Table 2, where the poisons are listed in descending order of lymphocyte toxicity, and the dose-effect curves are plotted in Fig. 2.

From these curves can be read off the LC at 50-48 hr for each poison (the concentration which kills 50 per cent of the lymphocytes in 48 hr). This, however, is not the exact lymphocyte LC_{50}, for by the time the cells were fixed and counted (48 hr) some of the dead lymphocytes had doubtless been removed by phagocytosis or autolysis, so the true LC_{50} must be somewhat higher than that read from these results. Nevertheless this qualified LC_{50} is a convenient yard stick with which to compare one poison with another. The LC_{50} figures are listed in column F of Table 1.

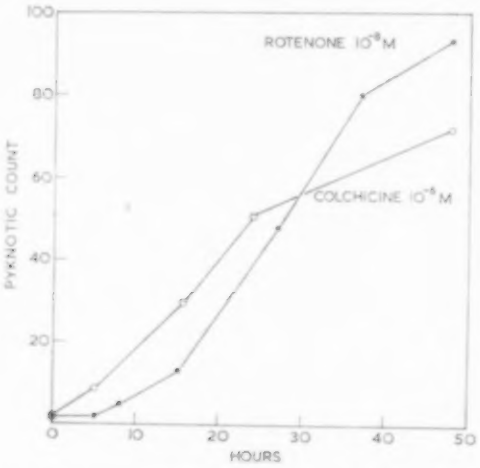

FIG. 1. Percentage of dead lymphocytes (pyknotic count) in lymph-node cultures at various time intervals after addition of colchicine or rotenone.

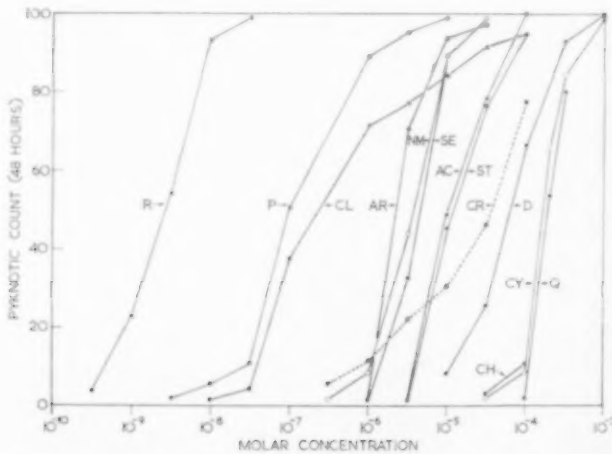

FIG. 2. Percentage of dead lymphocytes (pyknotic count) in lymph-node cultures 48 hr after addition of various concentrations of cell poisons: AC = acriflavine, AR = arsenite, CH = chelidonine, CL = colchicine, CR = cortisone, CY = cyanide, D = diphenylhydantoin, H = hydroquinone, NM = nitrogen mustard, P = podophyllotoxin, Q = quinone, R = rotenone, SE = selenite, ST = stilboestrol.

TABLE 2. PERCENTAGE OF DEAD LYMPHOCYTES (PYKNOTIC COUNT) IN LYMPH-NODE CULTURES 48 HR AFTER ADDING VARIOUS CONCENTRATIONS OF VARIOUS POISONS

(Each entry is the average value for from eighteen to twenty cultures. Control cultures (48 hr after no addition) = 1·2 per cent. F = cytological fixation. Percentage of reticulum cells dead indicated by: *<10, † = about 50, ‡ = nearly 100.)

Molar Concentration	3×10^{-10}	10^{-9}	3×10^{-9}	10^{-8}	3×10^{-8}	10^{-7}	3×10^{-7}	10^{-6}	3×10^{-6}	10^{-5}	3×10^{-5}	10^{-4}	2×10^{-4}	3×10^{-4}	10^{-3}
Rotenone	3·7*	22·7*	54·2*	93·4*	99·0†										
Podophyllotoxin			1·9*	5·4*	10·9*	50·4*		89·1*	95·2*	99·0†					
Colchicine				1·5*	4·3*	37·4*		71·9*	77·5*	84·5*	91·4†	94·6†			
Na arsenite								1·5*	70·9*	93·7*	97·2†				
N_2 mustard							1·7*	8·3*	44·1*	89·5*	99·0‡				
Na selenite								1·4*	32·7*	88·6*	100‡	F			
Acriflavine									1·3*	48·8*	78·5†	100†			F
Stilboestrol									1·2*	45·3*	76·7*	95·0‡			
Cortisone							5·4*	11·6*	22·1*	30·4*	46·1*	77·8*			
Diphenylhydantoin								2·0*		8·2*	25·5*	66·6*		93·1*	100‡
Chelidinine											2·6*	10·4*			
K cyanide											1·9*	8·5*	66·3†	84·6‡	99·1‡
Quinone												1·9*	53·8*	80·3†	F
Hydroquinone												2·5*	46·5*	80·4†	F

Table 2 shows also, by a system of *, †, ‡ scoring, the concentrations at which these various poisons killed the reticulum cells of the lymph node. Dead cells were recognized by nuclear pyknosis or karyolysis. There are always a few reticulum cells in the film preparations made from these cultures, enough to make this rough assessment but not enough for accurate counting. The results show that, except for cyanide which kills lymphocytes and reticulum cells about equally, all these poisons are selectively toxic to lymphocytes. In general, the concentration required to kill 50 per cent of the reticulum cells was at least ten times that needed to kill 50 per cent of the lymphocytes, and there was never any reticulum cell damage at the lymphocyte LC_{50} concentration.

Some of these poisons, in concentrations somewhat higher than those needed to kill lymphocytes, were found to act as histological fixatives. Acriflavine 10^{-3} M, hydroquinone 10^{-2} M and sodium selenite 10^{-4} M behaved in this way. In cultures exposed to these concentrations the lymphocytes and reticulum cells were all fixed, albeit rather poorly from a technical point of view, and there was of course no pyknosis. This fixative action was confirmed by fixing whole lymph nodes and thymus glands for 24 hr in corresponding solutions made up in isotonic saline, then embedding and cutting histological sections in the ordinary way. The histological structure was reasonably well preserved. Acriflavine is known to combine with and precipitate nucleic acids,[49] so its action is easily understood. Hydroquinone and selenite do not precipitate proteins or nucleic acids and their fixing action is difficult to explain. It may be recalled, however, that the fixing action of formaldehyde is similarly not understood.

DISCUSSION

The main object of this work was to find out if there is a universal parallelism between the antimitotic and the lymphotoxic effects of poisons acting in concentration of 10^{-5} M or less. In particular, is the relation sufficiently close to suggest a common underlying mode of action? The answer can be clearly seen in Fig. 3, and the two most important points emerging from the results are as follows.

(1) Virtually all the mitotic poisons known to be effective (on fibroblasts) at 10^{-5} M or less have been tested and all were found to be specifically toxic to lymphocytes at 10^{-5} M or less, except *quinone-hydroquinone* and *chelidonine*.

(2) In the course of other work (unpublished) we have investigated the toxicity of a very wide variety of chemicals to lymphocytes *in vitro* and all those exhibiting marked toxicity at 10^{-4} M or less are reported here in Table 2. Of these, all are mitotic poisons (on fibroblasts) at 10^{-5} M or less, except *selenite* and *diphenyhydantoin*.

So although there seems to be some sort of association between mitotic inhibition and lymphocyte poisoning, eight out of the twelve poisons tested having both actions, the association is not universal for there are two clear-cut exceptions either way. Quinone-hydroquinone, the most powerful mitotic poison known (for fibroblasts) is not toxic to lymphocytes at 10^{-4} M. Likewise chelidonine, a more powerful antimitotic than nitrogen mustard, is scarcely toxic to lymphocytes at 10^{-4} M. Contrariwise, selenite which is about as toxic to lymphocytes as nitrogen mustard and diphenylhydantoin which is almost as toxic as cortisone are not mitotic poisons.

On the other hand it must be pointed out that rotenone is both the most powerful lymphocyte poison yet recorded and also the most potent mitotic inhibitor of sea urchin eggs. And further, in the case of six poisons (rotenone, colchicine, arsenite,

nitrogen mustard, acriflavine, stilboestrol) the concentrations required for 50 per cent lymphocyte killing on the one hand and mitotic poisoning of fibroblasts on the other are practically the same.

It is remarkable that the chromosome poisons and the spindle poisons seem to be about equally good at killing lymphocytes. Now preventing the onset of mitosis would seem to be a very different proposition from destroying the spindle of a cell which has

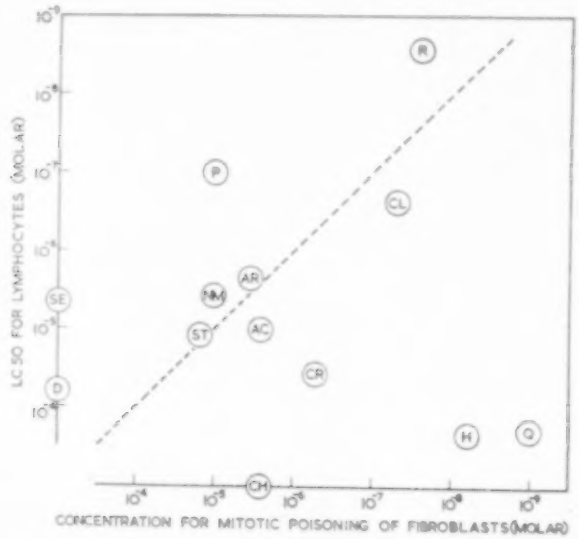

FIG. 3. Concentration which kills lymphocytes plotted against concentration which poisons mitosis of fibroblasts (both *in vitro*) for the various poisons. Lettering as for Fig. 2.

already passed through prophase, so much so that it is difficult to visualize any common biochemical lesion underlying such diverse phenomena as chromosome poisoning, spindle poisoning and lymphocyte killing. Taken as a whole, the results reported here would not support such a hypothesis. The wide chemical diversity of the effective poisons also speaks against it. If at this point we recall that ionizing radiation is also a mitotic inhibitor (acting like a chromosome poison) and a selective killer of lymphocytes, it is to point out that the task of extending the common biochemical lesion to include radiation as well is even more forbidding.

Such rough correlation as exists between mitotic poisoning and lymphocyte poisoning may be no more than a simple consequence of the fact that mitosis is the cell process and the lymphocyte the cell type most susceptible to adverse conditions.

On the general problem of the peculiar sensitivity of the lymphocyte to ionizing radiation and chemical poisons, the search for common chemical denominators seeming unfruitful, attention must be directed more to intrinsic features of the lymphocyte itself, particularly its metabolism. In this connexion in 1955 the writer[50] suggested that the relative paucity of mitochondria might be a significant point and subsequent measurements[51] showed the total mitochondrial volume of a lymphocyte to be 0·5–1·3 μ^3, compared with 800 μ^3 for a liver cell. Following this Creasey and Stocken[52]

showed that the nuclei of lymphocytes (and possibly some other radiosensitive cells) differed from those of other cells in that they had a phosphorylating system and could synthesize ATP, so they were normally not dependent on mitochondrial ATP. They also showed that this nuclear phosphorylating system was extremely sensitive to X-radiation. It is to specific differences in the intracellular localization of enzyme systems such as these that the sensitivity of the lymphocyte to radiation and mitotic poisons must ultimately be related.

It now remains to comment on some of the individual lymphocyte poisons. The three most powerful, rotenone, podophyllotoxin and colchicine are complex aromatic substances derived from plants. Rotenone is the active principle of the root of *Derris sp.* Originally used as a fish poison it is now widely used as an insecticide (derris dust). It is virtually harmless by mouth to warm-blooded animals,[5] probably because it is destroyed during digestion, but it can produce unpleasant symptoms when inhaled as a dust. Injected, it has about the same toxicity as cortisone (Table 1). Rotenone is the most powerful mitotic inhibitor known for sea urchin eggs (5×10^{-9} M) and it is here shown to be easily the most potent lymphocyte poison so far described. The toxicity of rotenone for lymphocytes is truly remarkable, it kills 20 per cent of cells at a concentration of 10^{-9} M, which, assuming a uniform distribution throughout cells and medium, amounts to only 100 molecules per cell. Prior to this discovery the lowest cytocidal concentration recorded for any chemical on any mammalian cell was 10^{-6} M for cortisone on lymphocytes.[8] Podophyllotoxin and colchicine are thirty times more active than this, while rotenone is a thousand times more active.

Leblond and Segal[54] reported that injection of colchicine produced great lymphoid atrophy in normal rats, but no effect in adrenalectomized ones. They therefore concluded that the lympholytic action of colchicine was entirely mediated by the adrenal cortex and that injection of colchicine was one of the most "alarming" stimuli known. The results obtained here show a direct effect of colchicine on lymphoid tissue at very low concentrations (10^{-7} M) and are difficult to reconcile with the findings of Lebond and Segal. The dose-effect curve for colchicine has a rather peculiar shape (Fig. 2) which seems to imply a hard core of lymphocytes which are relatively resistant to the drug. Murray *et al.*[55] showed that the metaphase arrest produced by colchicine in fibroblast cultures could be prevented or reversed by *m*-inositol. In our experiments, however, we found that the lymphocytocidal effect of 10^{-6} M colchicine was not prevented at all by 10^{-4} M *m*-inositol. It should perhaps be mentioned that the lymphocyte pyknosis produced in these experiments by colchicine and other spindle poisons was ordinary pyknosis and did not resemble the "clumped metaphases" produced in dividing tissues.

The figures for nitrogen mustard are in good agreement with those of Schrek who, working with lymphocyte suspensions *in vitro*, found the minimum concentration required to kill some cells to be 3×10^{-6} M for rabbit lymphocytes[56] and 6×10^{-6} M for human lymphocytes.[57]

The results obtained with quinone and hydroquinone were essentially identical, as has also been reported for the antimitotic action on fibroblasts[58] and sea urchin eggs[32]. This indicates that the effect on the cells is not produced by oxidation in the one case or reduction in the other. It also shows that the low toxicity found in these experiments cannot be accounted for by oxidation or reduction of the compound before it reached the cells.

The biochemical basis of selenite poisoning is still unknown. Although it has been shown to inhibit some SH enzymes *in vitro*[59] this cannot account for all its properties. SH reactants in general inhibit mitosis, but selenite does not. Selenite, arsenite and nitrogen mustard were about equally toxic to lymphocytes. Acriflavine and stilboestrol were almost identical and had about one third the toxicity of nitrogen mustard.

Diphenylhydantoin is a central nervous depressant used in the treatment of epilepsy. Clinical reports suggested that this drug may cause lymphopenia[60] and that is why it was investigated here. It was of further interest because we had previously shown[9] that barbiturates are selectively toxic to lymphocytes—but only in the range 10^{-4}–10^{-3} M which is rather higher than the concentration range considered here.

Acknowledgements—I am greatly indebted to Dr. Ivor Cornman of Hazleton Laboratories Inc., Falls Church, Va., U.S.A. for suggesting the trial of rotenone and for much information on the mitotic inhibition of sea eggs. I am grateful to Prof. J. S. Mitchell for drawing my attention to the lymphopenic action of diphenylhydantoin.

The culture work was carried out by Mr. W. R. Lush and Miss E. Peakman, and the cell counting by Mr. W. R. Lush. Miss M. Tebbutt made the film preparations and gave general assistance. It is a pleasure to thank these technicians for their very careful and painstaking work. I have to thank Messrs. Parke, Davis & Co. for a gift of pure sodium-5 : 5-diphenylhydantoin ("epanutin").

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Biochemical Pharmacology, 1960, Vol. 5, pp. 64–78. Pergamon Press Ltd., Printed in Great Britain.

THE METABOLISM OF 6-MERCAPTOPURINE BY *BACILLUS CEREUS**

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(*Received* 4 *January* 1960)

Abstract—The growth-inhibitory purine analog, 6-mercaptopurine, was oxidized by resting cells of *Bacillus cereus* to the non-inhibitory catabolites, 6-thioxanthine and 6-thiouric acid. In growing cell cultures other pathways of drug metabolism appeared to be more important. Sulfur was rapidly cleaved from the drug, could be recovered as inorganic sulfite, and appeared finally in the proteins. The resulting purine skeleton was incorporated as adenine and guanine into both ribo- and deoxyribo-nucleic acids. No direct evidence could be found that 6-mercaptopurine was incorporated as such into nucleic acids. The upper limit calculated for such incorporation suggests that this pathway of metabolism was most probably insufficient to produce growth inhibition. A compound having the properties of 6-mercaptopurine ribonucleotide was present in the soluble fractions of cells. Metabolism to non-inhibitory compounds, principally the normal purines of the nucleic acids, appeared to be responsible for the cessation of growth inhibition characteristically observed with 6-mercaptopurine. When the rate of growth returned to normal, all the 6-mercaptopurine had been removed from the growth medium. The failure of increasing concentrations of 6-mercaptopurine to increase the magnitude of growth-inhibition, in contrast to the observed increase in the duration of inhibitory action, has been discussed.

INTRODUCTION

THE purine analog 6-mercaptopurine has been of considerable interest since its introduction in the treatment of leukemias.[1] It has also been demonstrated to inhibit the growth of bacteria and of mammalian cells in culture.[2,3] The precise mechanism of action has not yet been elucidated, but the drug is known to interfere with the interconversion of purines in nucleic acid synthesis.[4,5] The possibility still exists that other biochemical pathways are affected also which may be even more closely related to the growth-inhibitory effect.

Studies on the metabolism of the drug in the mouse[6] and in *Streptococcus faecalis*[7,8] have revealed that some of the drug was converted to normal purines. Elion *et al.*[6] have suggested that the purine is incorporated into nucleic acids in mouse tissues, as is the case with certain other purine analogs.[9] In bacteria, however, such incorporation seemed to be slight.[10] The presence of 6-mercaptopurine ribonucleotide in microorganisms susceptible to the drug, in contrast to the absence of this ribonucleotide in resistant systems, has led Brockman *et al.*[11] to hypothesize that this anabolite is crucial in the drug's action. In the present study some of the routes by which the drug is metabolized have been investigated prior to studies on the mechanism of action of the

* This research was supported by Research Grant CY 2978 from the National Cancer Institute, U.S. Public Health Service, Bethesda, Maryland.

drug. The micro-organism *Bacillus cereus* was selected because its growth is partially inhibited by the drug and because this micro-organism has been the object of quantitative studies in this laboratory.[12] Some of the present work has been reported previously.[13,14]

MATERIALS AND METHODS

Compounds

6-Mercaptopurine was obtained from Burroughs Wellcome and Co., Tuckahoe, N.Y. 6-Mercaptopurine-8-^{14}C (0·55 mc/m-mole) and hypoxanthine-8-^{14}C (0·81 mc/m-mole) were purchased from Isotopes Specialties, Inc., Burbank, Cal. 6-Mercaptopurine-^{35}S (7·1 mc/m-mole) and 6-mercaptopurine ribonucleoside were gifts of Dr. R. Wallace Brockman, Southern Research Institute, Birmingham, Ala. Both radioactive samples of 6-mercaptopurine were found to be pure by chromatography. 2-Hydroxy-6-mercaptopurine (thioxanthine) was purchased from Mann Research Laboratories, New York 6, N.Y. 2:8-Dihydroxy-6-mercaptopurine (thiouric acid), synthesized by Dr. Ti Li Loo, National Institutes of Health, Bethesda, Md.,[15] was kindly furnished to us. Purified milk xanthine oxidase and catalase were obtained from the Worthington Biochemical Co., Freehold, N.J. Trypsin came from the Difco Laboratories, Detroit, Mich. *Crotalus adamanteus* venom was obtained from the Ross Allen Reptile Institute, Silver Springs, Fla.

Enzyme methods

To study the effect of xanthine oxidase on 6-mercaptopurine, 10 mg of the analog, 100 units of xanthine oxidase and about 0·2 mg of catalase were incubated in 1 ml of 0·025 M phosphate buffer of pH 7·5 at 37 °C for from 6 to 8 hr with occasional shaking.

To separate DNA from contaminating protein, 1—5 mg of the mixture was dissolved in 1·0 ml water, brought to pH 8·5, and 3 μg of trypsin was added; the mixture was incubated overnight at 37 °C, and the DNA reprecipitated as described below. Hydrolysis of nucleotides with *Crotalus* venom phosphatase was carried out by incubation at pH 9·0 for 6 hr, as described by Dunn and Smith.[16]

Micro-organism

Bacillus cereus, strain 569H, was grown in polyethylene bottles at 37 °C and was shaken to produce optimal aeration. The growth medium consisted of salts and a casein hydrolyzate (vitamin-free casamino acids, Difco Laboratories, Detroit, Mich.) adjusted to pH 7·0 with potassium hydroxide.[17] Growth was measured as change in turbidity, determined as optical density at 540 mμ in the Beckman spectrophotometer model DU. Usually, 6-mercaptopurine was added to exponentially growing cultures at an optical density reading between 0·05 and 0·06 (i.e. about 40 μg dry weight of cells per ml).

For the resting cell incubations, cells which had been grown in the salts–amino acids medium were centrifuged, washed in the medium from which the amino acids had been omitted, and suspended in this deficient medium in Roux bottles at 37 °C.

Isolation of nucleic acid components

The cells were fractionated by the alkali digestion procedure of Studel and Paiser,[18] as described by Schmidt and Thannhauser,[19] and extracted with hot saline solutions as described by Jorpes.[20]

After washing, the cells were treated with 5% trichloroacetic acid (TCA), and the precipitate was extracted with 70% ethanol at 70 °C, 95% ethanol at 70 °C, and ethanol: ether 3:1 (v/v) at room temperature. The RNA was then hydrolyzed with 1 N KOH for 18 hr. Alternatively, the cells were treated with TCA, the precipitate was suspended in ethanol, disintegrated with glass beads, and the nucleic acids were extracted by treatment of the residue three times with 10% NaCl at 100 °C for 1 hr.[21] The sodium nucleates were treated with TCA and 2 vols. of ethanol in the cold, and then hydrolyzed with potassium hydroxide as above. The DNA, protein and potassium ions were removed from the ribonucleotides by precipitation with perchloric acid at 0 °C, and the ribonucleotides were separated by paper electrophoresis. The DNA was isolated from the alkali digest by precipitation with 0·06 ml of 6 N HCl to 1 ml of digest and sufficient TCA to give a final concentration of 5 per cent. Two volumes of ethanol were then added and the mixture was stored overnight at −10 °C to ensure complete precipitation. The precipitate was centrifuged, washed with ethanol and dried. It was then treated with trypsin to remove remaining protein, as described above, and reprecipitated. The purines were removed from this fraction by a 5 min hydrolysis in 0·1 N HCl at 100 °C.[22]

Compounds were chromatographed on Whatman No. 3 MM paper, with *iso*propanol-H_2O (70:30 v/v) as solvent in an atmosphere of ammonia.[23] Material absorbing ultraviolet light was located on the chromatograms by ultraviolet light absorption prints according to the method of Markham and Smith,[24] using Remington Rand G-91 Portagraph paper. Radioactive spots were detected by placing the chromatogram against Kodak-Medical X-ray film (single coated, blue sensitive) for from 2 to 4 weeks. Compounds were separated by paper electrophoresis on strips of Whatman No. 3 MM paper, 2½ in. wide. A 1000 V power source (E.C. Apparatus Co., Swarthmore, Pa.) provided a potential gradient of approximately 20 V/cm. The strip was immersed in a bath of carbon tetrachloride placed between the electrode chambers, to prevent heating.[23] Solutions of 0·05 M formate, pH 3·5; 0·05 M acetate, pH 4·5 and 5·0; 0·05 M phosphate, pH 6·6 and 7·6; and 0·025 M borate, pH 8·5, 9·0 and 9·7 were used as buffers. The distribution of radioactivity and ultraviolet absorption along electrophoresis and chromatogram strips was measured by cutting the strips into 0·5 cm sections from which the compounds were eluted by immersion in 2 ml of 0·01 N HCl for 16 hr at 0 °C. After measurement of the ultraviolet absorption spectrum, 0·5 ml or 1 ml of each solution was transferred to an aluminum planchet, dried in a stream of cool air, and the radioactivity determined. Breakdown of some of the sulfhydryl compounds mentioned in this study has been observed to occur when the compounds were concentrated at one end of a chromatogram section by the capillary flow of an eluting fluid through the section, followed by evaporation from the end. This decomposition was avoided by immersing the spot in a large volume (10 ml or more) of water containing 1 drop of 1 N HCl in the cold, and lyophilizing the solution obtained.

Conversion of cysteine to cysteic acid

Cysteine in the bacterial protein fraction remaining after the sodium chloride extraction was oxidized to cysteic acid by a 5 min treatment with performic acid at room temperature.[25] The performic acid was removed by evaporation, and the protein hydrolyzed with 6 N HCl at 8 lb/in² (112 °C) for 3 hr. The cysteic acid was separated

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readily from the amino acid mixture by paper electrophoresis (2 hr at 20 V/cm, pH 3·0 (0·2 M acetic acid)), since under these conditions it is the only compound migrating towards the anode.

Membrane technique

The uptake of radioactive materials by growing cells was measured by the membrane filter technique of Britten *et al.*,[26] a method which has been used regularly in this laboratory.[12] Samples of the culture were rapidly transferred into Beckman cells for turbidity measurements, and were then pipetted immediately for filtration through collodion membrane filters (Schleicher and Schuell Co., Keene, N.H.). These filters, which removed the cells quantitatively from the cultures, were attached with rubber cement to aluminum plates and allowed to dry. The radioactivity on the plates was determined in a proportional gas-flow counter usually to a standard error of 1 per cent.

The above filtration technique has been used by Roodyn and Mandel[27] as a rapid method of fractionation which provides the same results as those obtained by conventional techniques. Bacteria were treated with 5% TCA for 15 min at room temperature, or with 5% TCA for 30 min at 100°C. These suspensions were filtered onto the membranes and the residues washed with 1% TCA. The radioactivity in the "intact cells" was determined by filtering the untreated culture directly and washing with medium.

Recovery of 6-mercaptopurine

The percentage of 6-mercaptopurine remaining in the growth medium after various periods of incubation was estimated by the following procedure. A sample of the medium containing 6-mercaptopurine-8-^{14}C, after measurement of turbidity, was filtered through a collodion membrane and the filtrate collected. Carrier 6-mercaptopurine (at least 2000 times greater than the amount of the radioactive compound) was added to an aliquot of this filtrate. The mixture was then lyophilized, the residue taken up in a small volume of 0·01 N NH_4OH, and the 6-mercaptopurine was re-isolated by a two-dimensional combination of paper chromatography and paper electrophoresis at pH 9·0. 6-Mercaptopurine was the only detectable spot on the paper which absorbed ultraviolet light after this treatment. The specific activity of this material, calculated from the optical density at 325 mμ, was directly proportional to the concentration of 6-mercaptopurine remaining in the growth medium, since the ultraviolet absorption of the radioactive material was insignificant in comparison with that of the carrier.

Preparation of soluble fractions

To prepare the soluble fraction of cells, 400 ml of culture was incubated with 1 μg of ^{35}S- or ^{14}C-6-mercaptopurine per ml of suspension. After 30–40 min, when the quantity of soluble metabolites was at a maximum, the culture was cooled in ice. The cells were centrifuged, suspended in water and then pipetted into 4 ml of ethanol at 85 °C. After 3 min, the precipitate was removed by centrifuging and the supernatant solution evaporated to dryness *in vacuo*.[28]

RESULTS

Metabolism of 6-mercaptopurine by resting cells

Resting cells of *Bacillus cereus* (about 2 mg of dry weight per ml of culture) were incubated in the presence and absence of 20 μg of 6-mercaptopurine-8-^{14}C per ml of

suspension for 15—40 hr at 37 °C. An ultraviolet absorption print and radioautogram of a chromatogram of the medium containing 6-mercaptopurine, after removal of cells, is shown in Fig. 1. Two spots absorbing ultraviolet light were observed, A and B, which were not formed during incubation in the absence of 6-mercaptopurine. Of the five radioactive spots, two coincided with A and B; two others, C and D, although they contained as much radioactivity as A and B, did not absorb ultraviolet light. The fifth radioactive metabolite, E, could be distinguished from an ultraviolet absorbing metabolite which was present also in the control.

Metabolite A had the same ultraviolet absorption spectrum and chromatographic properties as authentic thiouric acid. This compound has been reported to be a metabolite of 6-mercaptopurine in man[15,29] and in the mouse.[6] The radioactive metabolite which had been purified by paper chromatography was mixed with carrier thiouric acid from which it was subsequently indistinguishable upon electrophoresis at pH 4·5, 5·0, 6·6 and 7·6. The metabolite is therefore presumed to be thiouric acid.

Metabolite B had the same ultraviolet absorption spectrum and chromatographic properties as thioxanthine, a potential intermediate in the conversion to thiouric acid. When carrier thioxanthine was added to the bacterial medium after incubation with 6-mercaptopurine-8-^{14}C, the re-isolated thioxanthine had a constant specific activity

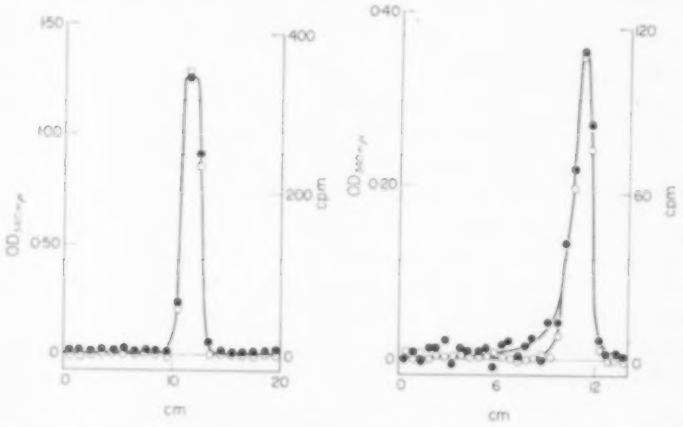

Fig. 2. Distribution of ultraviolet absorption (○——○) and radioactivity (●——●) from mixture of radioactive metabolite B and carrier thioxanthine. Left, paper chromatogram; right, paper electrophoresis strip, pH 7·6.

after the seventh and ninth recrystallizations from hot water. Since thioxanthine occasionally decomposed on recrystallization, reproducibility was improved by acidifying the incubation medium, lyophilizing and extracting the dry powder with hot pyridine. The pyridine was evaporated to dryness, and after carrier thioxanthine was added the mixture was recrystallized three times from hot water. Constant specific activity of the recrystallized thioxanthine was retained during chromatography followed by electrophoresis of the chromatographed compound at pH 6·6 and 7·6, as shown in Fig. 2, and therefore it was concluded that metabolite B is thioxanthine.

The high degree of sensitivity of the identification procedure shown in Fig. 2 was demonstrated by mixing radioactive thiouric acid, isolated from the medium, with carrier thioxanthine, and separating the mixture by electrophoresis at pH 6·6, at

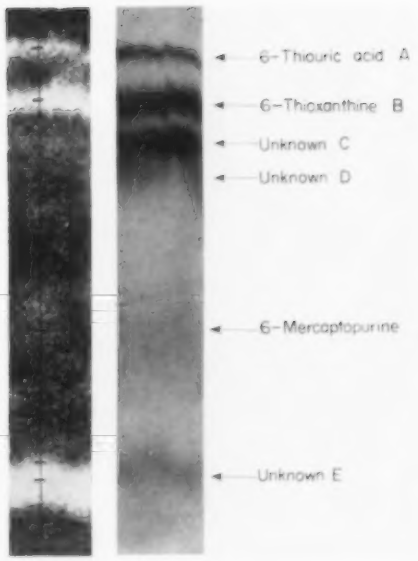

FIG. 1. Metabolites of 6-mercaptopurine-8-^{14}C in the medium of a resting *B. cereus* culture. Left ultraviolet absorption print and right, radioautogram of a chromatogram.

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which the compounds have similar mobilities. As shown in Fig. 3, the compounds could be differentiated from each other, since the specific activities of sections of the strip were not the same. Only a very slight difference in mobility is required, therefore, to distinguish a known compound from an unknown radioactive one.

Incubation of resting cells of *B. cereus* (2 mg of dry weight per ml) for 16 hr, in the presence of 20 μg/ml of thioxanthine led to the formation of a metabolite with the chromatographic and ultraviolet-absorbing properties of thiouric acid. Incubation

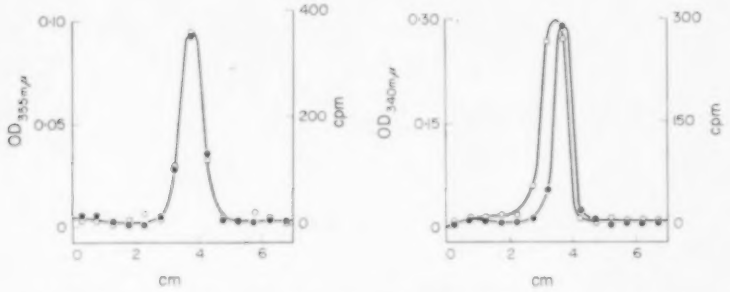

FIG. 3. Sensitivity of analysis method. Distribution of ultraviolet absorption (○——○) and radioactivity (●——●) along paper electrophoresis strips (pH 6·6). Left, radioactive metabolite A plus carrier thiouric acid; right, radioactive metabolite A plus carrier thioxanthine.

of 6-mercaptopurine with commercial xanthine oxidase, in the presence of catalase, gave rise to two products with the chromatographic and spectrophotometric properties of thioxanthine and thiouric acid. Table 1 gives an indication of the amount of the metabolites produced after two periods of incubation using the separation procedure shown in Fig. 1. Metabolite C was not distinguished clearly from thioxanthine in this experiment. It appears that thiouric acid was produced at a slower rate than

TABLE 1. RADIOACTIVE METABOLITES OF 6-MERCAPTOPURINE-8-^{14}C IN THE MEDIUM OF RESTING *B. cereus* CULTURES

Compound	Code	Per cent of total radioactivity	
		16-hr incubation	40-hr incubation
6-Thiouric acid	A	5	33
6-Thioxanthine + unknown	B + C	50	42
Unknown	D	—	4
6-Mercaptopurine	—	42	12
Unknown	E	3	9

thioxanthine, consistent with the hypothesis that 6-mercaptopurine was converted first to thioxanthine, which was subsequently oxidized to thiouric acid, Neither thioxanthine nor thiouric acid was found to inhibit the growth of *B. cereus* at concentrations up to 20 μg/ml. Since 6-mercaptopurine is inhibitory to growth at less than one-twentieth of this concentration, this metabolic route apparently involved detoxication. The three other metabolites produced by resting cells remain unidentified.

Metabolism of 6-mercaptopurine by growing cells

A dose-response curve with 6-mercaptopurine (Fig. 4) demonstrated that the generation time of *B. cereus* was essentially constant during the inhibition of growth at all the levels tested, whereas the duration of inhibition was related to the drug concentration. Similarly, Bolton and Mandel[10] have reported that the inhibition of the growth of *E. coli* by 6-mercaptopurine terminated spontaneously after some time, and

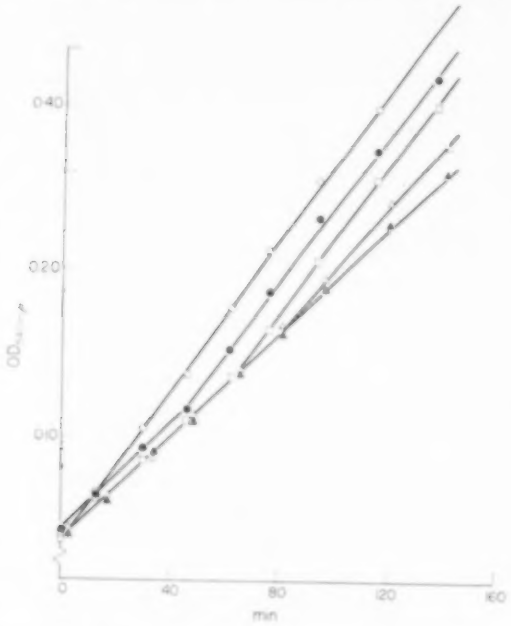

FIG. 4. Effect of various concentrations of 6-mercaptopurine on the growth rate of *B. cereus*. ○——○, 0 μg/ml; ●——●, 0·5 μg/ml; □——□, 1·0 μg/ml; △——△, 2·0 μg/ml; ▲——▲, 5·0 μg/ml.

normal growth resumed. Since metabolism of the drug could have been responsible for this cessation of inhibition, the metabolic pathways of 6-mercaptopurine were studied with growing bacteria. The conversion to thioxanthine and thiouric acid appeared to be of little importance in growing cells, since after 10 hr of growth in the presence of an excessive dose of 6-mercaptopurine (50 μg/ml) less than 10 per cent of the radioactivity remaining in the bacterial medium was recovered in the form of these metabolites.

Cells growing in the presence of 1 μg of either 6-mercaptopurine-8-^{14}C or 6-mercaptopurine-^{35}S per ml of medium were fractionated by the membrane technique. Fig. 5 shows the incorporation of the radioisotopes into the various fractions of the growing cells. It is clear that the fate of the two radioactive isotopes was quite different, implying that the sulfur was rapidly cleaved from the purine ring. Radioactive carbon was largely incorporated into the fraction soluble in hot TCA, which contained all of the nucleic acids. ^{35}S from 6-mercaptopurine-^{35}S, on the other hand, was incorporated in the residue insoluble in hot TCA, which contained essentially all the protein of the cell,[27] and only a small amount of radioactivity could be extracted with hot TCA. The relatively high incorporation of radioactive carbon into the hot TCA-insoluble

residue soon after the addition of the drug was observed repeatedly. Although the nature of this substance is unknown, it is tempting to speculate that it may represent a purine which is closely associated with protein. The proportion of radioactivity from the carbon-labeled drug in the acid-soluble fraction was much smaller than that of the ^{35}S from the sulfur-labeled drug, a finding which implies that the removal of the sulfur was rapid and extensive. The decrease in the total radioactivity inside the cells, particularly with the ^{35}S-labeled drug, was apparently due to the elimination of metabolites from the cell into the medium after the splitting of the sulfur-purine bond.

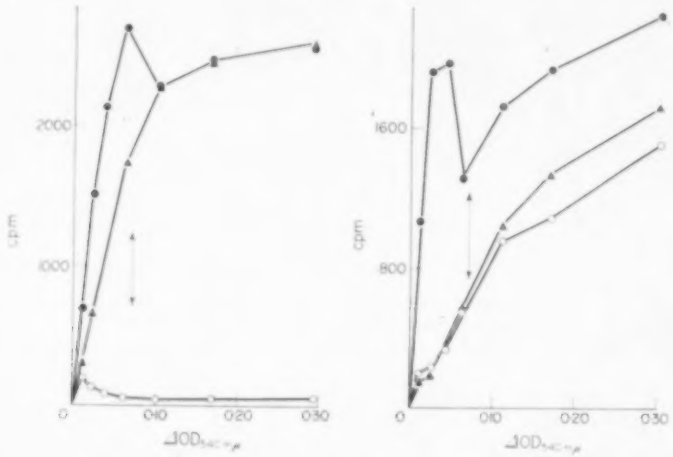

FIG. 5. Uptake of radioactivity from labeled 6-mercaptopurine by growing cells of *B. cereus*. Left, 8-^{14}C; right, ^{35}S. ●——● intact cells; ▲——▲ residue after treatment with cold TCA; ○——○ residue after treatment with hot TCA. Inhibition stopped at arrow.

Fig. 5 demonstrates that when the growth inhibition ceased, radio-carbon was no longer present in the acid-soluble fraction, while the radioactivity in the residue insoluble in cold TCA remained constant. However, radioactive sulfur continued to be incorporated into all fractions at approximately the same rate, even after the inhibition had ceased. It is interesting that the initial phase of uptake of radiosulfur into the acid-soluble fraction had been completed prior to the cessation of inhibition.

The decrease in the concentration of 6-mercaptopurine in the growth medium during incubation was estimated after various periods of incubation, using the procedure described under Methods. The cells were grown with 6-mercaptopurine-8-^{14}C at an initial concentration of 0·5 μg/ml. As is shown in Fig. 6, 6-mercaptopurine could no longer be detected in the medium at a time which coincided with the cessation of inhibition of cell growth.

Metabolites of 6-*mercaptopurine*-8-^{14}C *in the nucleic acid fraction.* The mononucleotides derived from the RNA of cells which had been incubated for 2 hr in the presence of 2μg of 6-mercaptopurine-8-^{14}C per ml were separated by paper electrophoresis at pH 3·5. Fig. 7 shows the distribution of ultraviolet absorption and radioactivity along the electrophoresis strip. Two major radioactive peaks were observed which coincided exactly in position with adenylic and guanylic acids and had the same ultraviolet absorption spectra. It was calculated that approximately 55 per cent of the

radioactivity from 6-mercaptopurine-8-^{14}C incorporated into the nucleic acids was present as adenine and 45 per cent as guanine. A small additional peak corresponding to another compound was also observed at about 8·5 cm from the origin. This product lacked the characteristic absorption at 320 mμ of a 6-mercaptopurine derivative, and its mobility was less than that expected for 6-mercaptopurine ribonucleotide. Since such a

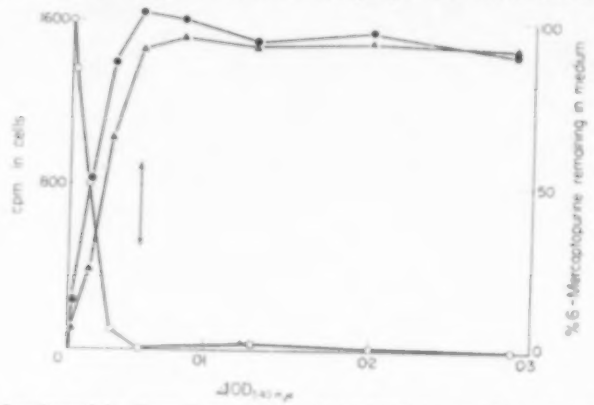

FIG. 6. Uptake of radioactivity from 6-mercaptopurine-8-^{14}C into growing cells of *B. cereus*; intact cells ●——●; residue after treatment with 5% TCA ▲——▲. Percentage of original 6-mercaptopurine remaining in the growth medium ○——○. Growth inhibition stopped at arrow.

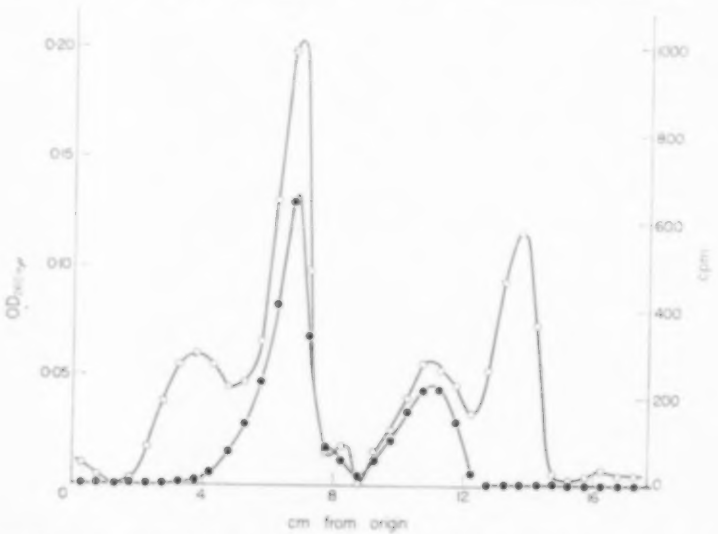

FIG. 7. Distribution of ultraviolet absorption (○——○) and radioactivity (●——●) along paper electrophoresis strip. Ribonucleotides from RNA of cells incubated with 6-mercaptopurine-8-^{14}C separated at pH 3·5.

compound has also been observed in experiments with labeled hypoxanthine, it was concluded that the product was derived from 6-mercaptopurine only after the cleavage of the mercapto group.

To determine whether or not 6-mercaptopurine was converted into the purines of DNA, bacteria were grown in the presence of 6-mercaptopurine-8-^{14}C, as is described

above, and DNA was isolated. After hydrolysis to obtain the purine bases, carrier 6-mercaptopurine was added and the mixture was subjected to electrophoresis at pH 9·7. Adenine and guanine were found to be radioactive, whereas no radioactivity could be detected in the 6-mercaptopurine area of the strip. Therefore, the presence of radioactivity from 6-mercaptopurine-8-^{14}C in the fraction soluble in hot TCA may be attributed to the conversion of the drug to adenine and guanine derivatives.

For purposes of comparison, bacteria were incubated for 1 hr with hypoxanthine-8-^{14}C at a concentration of 5 μg/ml and the RNA fraction was hydrolyzed and subjected to electrophoresis as before. The distribution of radioactivity along the paper strip was very similar to that shown in Fig. 7. Of the radioactive material in the nucleic acids, 60 per cent was recovered in the adenine and 40 per cent in the guanine components.

The incorporation of ^{35}S *from 6-mercaptopurine-*^{35}S *into the protein fraction.* The distribution of ^{35}S between the various cell fractions indicated that most of the radioactivity was incorporated into the proteins (Fig. 5). Cells which had been grown in the presence of 3 μg of 6-mercaptopurine-^{35}S per ml of culture for 2 hr were harvested and extracted to prepare the protein residue. The protein was treated with performic acid to oxidize the cysteine to cysteic acid and was then hydrolyzed. Carrier cysteic acid was added and the amino acid mixture was separated by paper electrophoresis. The isolated cysteic acid was radioactive, implying that the sulfur from the drug had been used by the cells in the synthesis of proteins. It seems reasonable to suppose that all the radio-sulfur in the residue insoluble in hot TCA was present as the sulfur amino acids formed from the sulfur split from 6-mercaptopurine.

Metabolites of 6-mercaptopurine-^{35}S *in the nucleic acid fraction.* Since a trace of radioactive sulfur was solubilized during the extraction of the nucleic acid fractions, attempts were made to establish an upper limit of the possible incorporation of 6-mercaptopurine-^{35}S as such into the nucleic acids. The extraction with hot TCA is known to remove more than nucleic acids alone, which do not contain sulfur. For instance, from *B. cereus* grown in the presence of cystine-^{35}S, about 10 per cent of the cellular radioactivity is extracted by this reagent.[30]

The mononucleotides derived from the RNA of a culture grown for 2 hr in the presence of 6-mercaptopurine-^{35}S at a concentration of 3 μg/ml, were chromatographed and the radioactivity along the paper was measured. In the areas to which the nucleotides migrated, two small unidentified peaks of radioactivity were observed. By assuming that both of these compounds contained 6-mercaptopurine itself, even though they were probably derived from contaminating non-nucleic acid material, and from a knowledge of the specific activity of the 6-mercaptopurine-^{35}S, a maximum of 2×10^{-4} μmole of the drug could have been associated with 0·5 μmole of RNA guanine, or one residue of 6-mercaptopurine for 2500 of guanine. In all likelihood, therefore, there would be less than 1 mole of drug present in 10,000 normal ribonucleotide units, or in RNA having a molecular weight of 3×10^6.

The incorporation of 6-mercaptopurine-^{35}S into DNA also was examined, si ance trace of radioactivity was associated with this fraction. Treatment with trypsin of DNA prepared from cells incubated as above with 6-mercaptopurine-^{35}S reduced the radioactivity in the precipitable material by about 60 per cent, presumably due to the release of soluble peptides. After hydrolysis to liberate the purines, carrier 6-mercaptopurine was added and the mixture was subjected to paper electrophoresis

at pH 9·0. Since the 6-mercaptopurine area of the strip differed in radioactivity from that of the background by less than 3 counts/min, it was calculated that no more than $1{\cdot}6 \times 10^{-5}$ μmole of 6-mercaptopurine could have been present. Since the strip contained 0·157 μmole of guanine, the incorporation of 6-mercaptopurine did not exceed 1 mole for each 10,000 moles of guanine, or one 6-mercaptopurine residue for every 40,000 normal base residues.

Stability of 6-mercaptopurine in alkali. It was observed that 6-mercaptopurine is considerably less stable in alkaline than in acid solutions. The possibility existed that the digestion with potassium hydroxide used in the above experiment destroyed 6-mercaptopurine, thus precluding the possibility of detecting the original drug or its anabolites. After incubation of 6-mercaptopurine or its ribonucleoside in 1 N potassium hydroxide for 16 hr at room temperature, chromatography revealed a single ultraviolet-absorbing component, the spectrum of which was identical to that of the untreated material. Thus, it seems reasonable to assume that any 6-mercaptopurine ribonucleotide present in the nucleic acids would have been similarly unaffected by the digestion.

Metabolites in the soluble fraction of the cells. Curve 1 of Fig. 8 shows the distribution of radioactivity along a chromatogram of the metabolities in the soluble fraction from cells incubated with 6-mercaptopurine-^{35}S, as described in Methods. Since, by analogy with the metabolism of cysteine,[31] sulfite may be expected to be a metabolite of 6-mercaptopurine, the soluble fraction was dissolved in water, and 10 mg of sodium sulfite and excess hydrochloric acid were added. The mixture was then allowed to stand in a closed container near an open dish of barium acetate solution to trap volatile sulfur dioxide. The resulting barium sulfite precipitate was isolated and was found to be radioactive. Chromatography of the soluble fraction which had been subjected to the above acid-treatment indicated that the large slow-moving radioactive peak was reduced in size (Fig. 8 curve 2).

To exclude the possibility that the extract contained radioactive metabolites other than sulfite which would be rendered volatile by acid, the ethanol extract derived from cells incubated with 6-mercaptopurine-^{35}S was divided into two equal portions. One portion was evaporated to dryness and 0·05 ml of saturated sodium bisulfite solution was added, followed by an equimolar amount of benzaldehyde. The benzaldehyde–bisulfite complex which formed was recrystallized from ethanol or *iso*propanol, and was found to be radioactive. To the second portion of the ethanol extract, 10 mg of sodium bisulfite and excess hydrochloric acid were added and sulfur dioxide was allowed to distill into barium acetate, as is described above. The solution was then evaporated to dryness and the benzaldehyde–bisulfite complex formed. The total radioactivity of this complex was considerably lower than in the first case. The amount of radioactivity lost from the extract by treatment with acid was essentially constant when determined in three ways (Table 2): (1) by direct counting of the radioactivity in the extract before and after the acid treatment; (2) by determining the amount of activity in the barium sulfite precipitate; and (3) by calculation from the difference in specific activities of the benzaldehyde bisulfite complexes formed with and without acid treatment. These results imply that inorganic sulfite was one of the metabolites of 6-mercaptopurine-^{35}S. In view of the probable reutilization of some of the sulfite for protein synthesis, an exact estimate of the extent of this desulfuration at any one time, as in the sampling procedure described here, would have little significance.

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The presence of 6-mercaptopurine ribonucleotide was suggested by the work of Brockman *et al.*[11] and of Paterson.[32] In the present experiments, the chromatographic distribution of radioactive metabolites in the soluble fraction was compared using cells incubated with 6-mercaptopurine-^{35}S (curve 1, Fig. 8) and 6-mercaptopurine-8-^{14}C

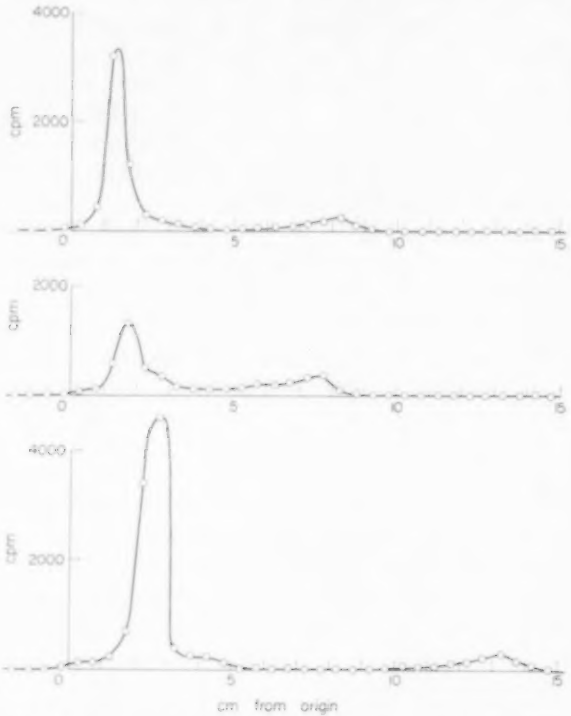

FIG. 8. Distribution of radioactivity along chromatograms of the soluble fraction of cells. Curve 1 (top), cells incubated with 6-mercaptopurine-^{35}S; curve 2 (center), cells incubated with 6-mercaptopurine-^{35}S after removal of sulfite; and curve 3 (bottom), cells incubated with 6-mercaptopurine-8-^{14}C.

TABLE 2. RADIOACTIVITY DERIVED FROM THE SOLUBLE FRACTION OF *B. cereus* AFTER INCUBATION WITH 6-MERCAPTOPURINE-^{35}S

(Acid-treatment volatilized sulfite as sulfur dioxide which was trapped as barium sulfite.)

	Radioactivity (counts/min)		
	Before acid-treatment	After acid-treatment	Volatilized by acid-treatment
Total activity of extract	58,150	49,830	8320
Total activity of barium sulfite precipitate	—	8060	8060
Total activity of benzaldehyde-bisulfite complex	14,640	4440	10,200

(curve 3, Fig. 8). The major peaks in both fractions had a low R_f (1·5–3·5 cm from origin), which is characteristic of nucleotides. These peaks were eluted and resolved by paper electrophoresis at pH 3·5. Apart from material remaining at the origin, there was only one other peak which coincided exactly in both paper strips. The properties of this material (low R_f, high electrophoretic mobility at pH 3·5, labeled with both ^{35}S and ^{14}C) were similar to those expected of 6-mercaptopurine ribonucleotide.

For the identification of the ribonucleotide of 6-mercaptopurine, the major peak from curve 2 of Fig. 8 (cm 1·5–3·5) was eluted and treated with *Crotalus* venom to hydrolyze any ribonucleotides present to the corresponding ribonucleoside.[16] A known amount of authentic 6-mercaptopurine ribonucleoside was added as carrier and the mixture was chromatographed in the *iso*propanol–ammonia solvent, in which ribonucleosides have a higher R_f than ribonucleotides. The ribonucleoside was eluted and subjected to paper electrophoresis using borate buffers at pH 8·3 and 9·0. The specific activities of sections of the ribonucleoside spot were constant along the length of the strip. It was concluded that the material which had a low R_f in curve 2 of Fig. 8 contained 6-mercaptopurine ribonucleotide. Assuming 100 per cent hydrolysis by the phosphatase, about one-fifth of the total radioactivity of the soluble fraction was associated with the ribonucleotide, or about 1 μmole of the metabolite per g of dry weight of cells.

DISCUSSION

The conversion of 6-mercaptopurine to thiouric acid in resting cells suggested that the enzyme xanthine oxidase was partly responsible for the metabolism of the drug, as had been demonstrated earlier for mammalian cells.[6,15,29] The similarity to the oxidation of hypoxanthine by this enzyme has been further demonstrated here with the observation that 6-mercaptopurine is converted by resting cells first to thioxanthine, which is then oxidized to thiouric acid. Thioxanthine has recently been shown to be a metabolite of thioguanine in mammalian tumor cells.[33]

This katabolic pathway is characteristic only of resting cells. Growing cells rapidly converted most of the drug into normal purines which were incorporated subsequently into nucleic acids. Thus, after a brief period of incubation, very little of the original compound remained to be degraded by the route characteristic of resting cells.

This conversion to normal purines is of importance, since a number of studies have suggested[34] that 6-mercaptopurine inhibits purine synthesis and purine interconversions because of an observed decrease in incorporation of a radioactive precursor into the nucleic acids or a lowering of the specific activity of the purines in the nucleic acids. It is clear that at least some of these decreases could be due to a dilution of the radioactivity from the precursor with non-radioactive purines derived from the drug. In those studies in which the total amount of nucleic acids was measured, however,[10,35] an actual decrease in nucleic acid content was shown to have been caused by the drug. Furthermore, since normal purines will reverse the growth inhibition, the metabolism of the drug itself produces compounds which will tend to overcome the inhibition.

Radioactive sulfur which had been cleaved metabolically from 6-mercaptopurine was lost rapidly into the growth medium, but later it re-entered the cell and was incorporated into the protein from which some of it was recovered as cysteic acid. Sulfite, which was concluded to be a product of metabolism of the drug, was present in the soluble fraction of the cells. It is possible that in the cell 6-mercaptopurine was

converted to purine-6-sulfinic acid which was then split to give sulfite and hypoxanthine. Since some of the radioactivity from cells grown in the presence of ^{35}-S-amino acids which are incorporated mainly into proteins can be extracted by procedures which extract principally nucleic acids,[30] caution must be exercised in deciding whether or not radioactive sulfur from 6-mercaptopurine-^{35}S which is liberated during such extraction is, in fact, due to incorporation of the unchanged drug.

The results presented indicate that little if any 6-mercaptopurine was incorporated into the nucleic acids of *B. cereus*, in agreement with earlier investigations with *E. coli*,[10] but apparently at variance with those on animal tissues.[6] The upper limit of incorporation was calculated to be less than one molecule of 6-mercaptopurine in a molecule of RNA having a molecular weight of 3×10^6. It seems unlikely that such a low level of fraudulent nucleic acid, containing the unnatural base, could account for the biological activity of the drug, at least in micro-organisms, especially since inhibition of growth by 6-mercaptopurine begins almost immediately (which, therefore, would not allow for any such incorporation into nucleic acids) and ceases abruptly when all the drug has been metabolized to other compounds.

An alternative suggestion, namely that 6-mercaptopurine inhibits growth by interfering with cell metabolism at the nucleotide level, is supported here by the identification of 6-mercaptopurine ribonucleotide in the soluble fraction, as in the work of Brockman *et al.*[36] with *Streptococcus faecalis* and by Paterson[32] with a tumor. Since inability to synthesize this ribonucleotide has resulted in resistance to 6-mercaptopurine,[36] it has been suggested that the ribonucleotides of the unnatural purines may be, or are closely related to, the true inhibitors of metabolism and therefore of growth.

It is clear that the relatively short duration of inhibition of *B. cereus* (also observed in *E. coli* by Bolton and Mandel[10]) is due to the metabolism of the drug to inactive compounds. In the initial stages of inhibition sufficient 6-mercaptopurine is present to allow production of the inhibitory metabolite. Thereafter, the competition between these anabolic processes and the catabolism which results in the formation of normal purines may antagonize the inhibitory action of the drug, and reduce the concentration of 6-mercaptopurine below that required for inhibition of growth. Thus, the duration of inhibition produced by decreasing concentrations of 6-mercaptopurine will be shorter as bacterial enzymes can catabolize the smaller quantities of the drug more readily.

Since increasing concentrations of the drug in the bacterial medium did not produce greater inhibition, it is possible that the enzyme systems which produced the inhibitory metabolite became saturated. Concentrations as low as 0·1 μg of 6-mercaptopurine per ml were definitely growth inhibitory for short periods of time and thus must have been adequate to saturate this enzyme system.

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Biochemical Pharmacology, 1960, Vol. 5, pp. 79–86. Pergamon Press Ltd. Printed in Great Britain.

CORRELATION OF THE METABOLIC EFFECTS OF BENZALKONIUM CHLORIDE WITH ITS MEMBRANE EFFECTS IN YEAST*

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(*Received* 22 *March* 1960)

Abstract—To non-growing suspensions of baker's yeast at pH 5·5 were added varying amounts of the cationic surfactant, benzalkonium chloride. The percentage of cells stained by Nile Blue dye in the presence of calcium chloride, the percentage of potassium lost from the cells, the percentage inhibitions of rates of anaerobic glucose utilization and CO_2 production, and the decarboxylation of exogenous pyruvate (as percentage of maximal rate of decarboxylation) were found to be equal for any given surfactant concentration in the range of concentrations used. Results were interpreted as an all-or-none phenomenon for individual cells in suspension, i.e. as the concentration of surfactant was raised, greater numbers of cells simultaneously released all or nearly all of their potassium, ceased fermenting, and commenced to decarboxylate pyruvate. The results are discussed.

INTRODUCTION

CATIONIC surface-active agents produce changes in both membrane permeability and metabolism. Hotchkiss[1] reported the loss of phosphorus- and nitrogen-containing compounds from staphylococci treated with cationic surfactants. Armstrong[2] showed a correlation between the cytolytic effects of cationic surfactants and their inhibitory effects on acid production and CO_2 production in yeast.

Poisons other than surfactants are known to increase the permeability of the red blood cell[3, 4] or of the yeast cell[5] in an all-or-none manner. Thus, each cell in suspension shows no membrane response until the concentration of agent becomes critical for that cell; at the critical concentration the membrane permeability to potassium suddenly increases with a resultant complete loss of this ion from the cell. The cells in a suspension would behave as a population, with greater numbers of cells losing their potassium as the concentration of agent was increased.

The aforementioned findings suggested that both the membrane response and certain metabolic responses of yeast to the cationic surfactant, benzalkonium chloride,† might be of an all-or-none nature. Results reported here lead to the conclusions that the effects of benzalkonium chloride on the membrane and on certain metabolic measurements are indeed all-or-none in nature. (In this paper the effects of the surfactant on the membrane were measured in terms of K^+ loss, but it is not implied that K^+ loss is the only result of alteration in the membrane produced by this agent.)

* This work was supported by USPHS Grant No. E-2234.

† Benzalkonium chloride, a mixture of cationic quaternary ammonium derivatives of varying alkyl chain lengths was supplied by Mr. Irwin Shupe of Winthrop Laboratories.

METHODS

All experiments were performed at pH 5·5 on non-growing suspensions of commercial baker's yeast (standard brands). The yeast had been washed four times and aerated for 4 hr prior to experiment. Manometric measurements of oxygen consumption or CO_2 production were made by the usual Warburg techniques.

Concentration of potassium ion in supernates from the suspensions was determined by flame photometry with lithium as internal standard. Total cellular potassium was estimated by boiling an aliquot of the yeast suspension for 2–3 min[6] and measuring the potassium in the supernate.

The dye, Nile Blue, was used to determine whether or not surfactant affected the membrane in an all-or-none manner. It was found that as surfactant concentration was raised, greater numbers of cells were stained. In any particular count there appeared only two types of cells—cells deeply stained and cells apparently unstained. The cells were protected from the effects of the dye by previous addition of calcium chloride to give a final calcium concentration of 0·005 M. The concentration of dye in the suspensions was 0·037 mM. At this dye concentration 97–100 per cent of the cells were stained when calcium chloride was not present. In the presence of calcium, however, the staining was reduced to 0–3 per cent. However, cells which have undergone membrane changes stain even in the presence of calcium. The effects of benzalkonium chloride on potassium loss and staining could thus be determined by adding surfactant to a yeast suspension, allowing the suspension to stand for 30 min with occasional shaking, withdrawing aliquots for potassium analysis, and then adding $CaCl_2$ and dye to other aliquots. After 45 min the aliquots containing the dye were then placed in a hemocytometer and the percentage of stained cells was determined.

A commercial glucose oxidase (Glucostat-Worthington Biochemical Corp.) preparation was utilized for glucose analysis. Alcohol was determined by use of a commercial alcohol dehydrogenase preparation (Determatube C-alc-Worthington Biochemical Corp.)

In experiments where pyruvate was used as substrate, acetaldehyde was determined by a modified Barker–Summerson[7] method for lactate. Apparently acetaldehyde, and not lactate, was being produced in these experiments, since zinc lactate standards required an initial 5-min heating period to convert the lactate to acetaldehyde. Barker and Summerson[7] also found the 5-min heating period to be necessary for conversion. The supernates from the pyruvate experiments, however, yielded acetaldehyde-like material without the initial 5 min of heating. An independent check for acetaldehyde was made by means of a modification of the method of Friedemann, *et al.*[8] Material in the supernates was volatilized at room temperature and reduced pressure, trapped in sodium bisulfite, and titrated by the method of Friedemann *et al.*[8] The apparent levels of acetaldehyde were quantitatively similar to, though more variable than, those obtained by the modified Barker–Summerson method just described.

Effects of benzalkonium chloride on the cell membrane as measured by net potassium loss

In Fig. 1 is shown a plot of potassium ion concentration in the medium as a function of time for various surfactant concentrations. Since no K^+ was added to the suspensions, any K^+ found in the suspending medium had to come from the cells. With the exception of the suspension containing the highest concentration of surfactant, most of the K^+ that was going to be lost from the cells had been lost by the time the first

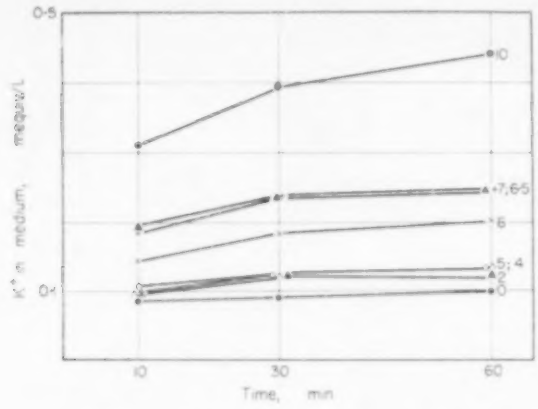

FIG. 1. Leakage of potassium vs. time, for different concentrations of benzalkonium chloride. Yeast concentration was 5 mg/ml wet weight. Suspensions were buffered at pH 5·5 with 0·03 M tris-succinate tartarate made by adding solid tris-(hydroxymethyl) aminomethane to the acids until appropriate pH was reached. Numbered curves represent mg of surfactant/g yeast. One experiment.

samples (10 min) were taken. In the time interval 30–60 min the further loss of K^+ was slight. In Fig. 2 the data of Fig. 1 are replotted to show net K^+ loss vs. surfactant concentration. It can be seen that K^+ loss increased slightly until a surfactant concentration of 0·004–0·005 (mg surfactant/mg yeast) was reached. Above this concentration the net K^+ loss rose sharply until it amounted to about 90–100 per cent of the total cellular K^+ at 0·010 surfactant concentration. Although in Figs. 1 and 2 the results for

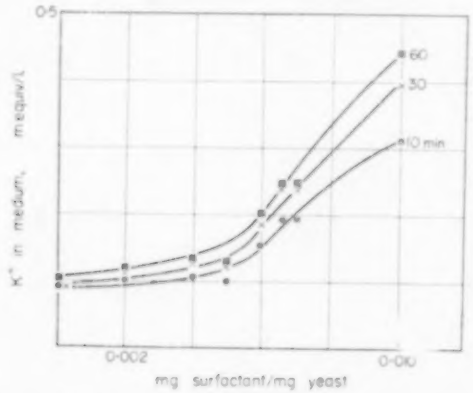

FIG. 2. Potassium leakage as a function of benzalkonium chloride concentration (replotted from Fig. 1).

only one experiment are shown, the K^+ loss in ten experiments was similar to that shown in the figures and was independent of the presence of, or the type of, substrate.

Because the response of individual cells could not be ascertained from the data of Figs. 1 and 2, the staining technique with Nile Blue in the presence of calcium was used to show individual cell response to surfactant. Calcium chloride and Nile Blue dye were added to suspensions previously subjected to the action of surfactant (see

Methods). It was assumed that the cells in the population absorbed dye as the result of an increased permeability brought about by surfactant. Results for net K^+ loss (as percentage of total cellular K^+) and percentage staining are shown in Fig. 3. It is evident that the percentage K^+ loss and percentage cells stained could be represented

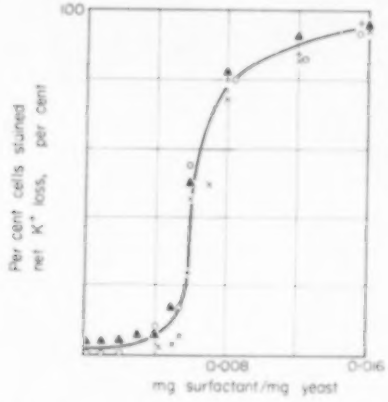

FIG. 3. Simultaneous staining and potassium loss for different concentrations of benzalkonium chloride. Total potassium loss was corrected for the control potassium loss. Yeast concentration and buffer same as in Fig. 1. See text for procedure. Two experiments. Symbols: + and ×: % staining. ▲ and 0: % net K^+ loss.

by the curve shown. In Fig. 4 the data of Fig. 3 and of two other experiments are plotted to show the variation of the experimental points from the line representing equal percentage K^+ loss and percentage staining. From Fig. 4 it can be seen that the percentage K^+ loss and percentage staining were approximately equal for each surfactant concentration. These data are best explained as representing an all-or-none phenomenon.

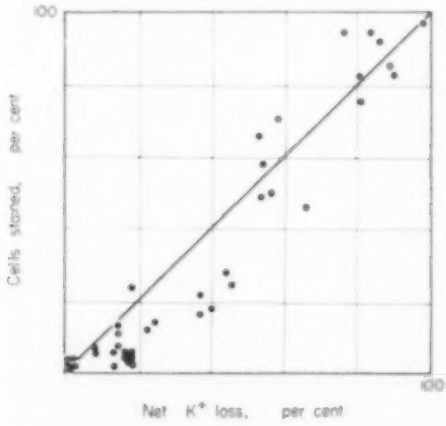

FIG. 4. Relationship of staining to potassium loss due to surfactant. Conditions same as in Fig. 3. Benzalkonium chloride concentration: 0–16 mg/g yeast (wet weight). Values corrected for control potassium loss. Four experiments.

Effects of benzalkonium chloride on metabolism

Decarboxylation of pyruvate. In confirmation of the findings of Barron *et al.*[9] it was found in the present study that intact yeast fails to metabolize pyruvate under anaerobic conditions at pH 5·5. At this pH the negatively charged pyruvate ion cannot penetrate the cell membrane.[9, 10] Because of the ability of benzalkonium ion to affect membrane permeability, it was decided to study the effects of surfactant on the metabolism of exogenous pyruvate. In Fig. 5 it is seen that decarboxylation of pyruvate by

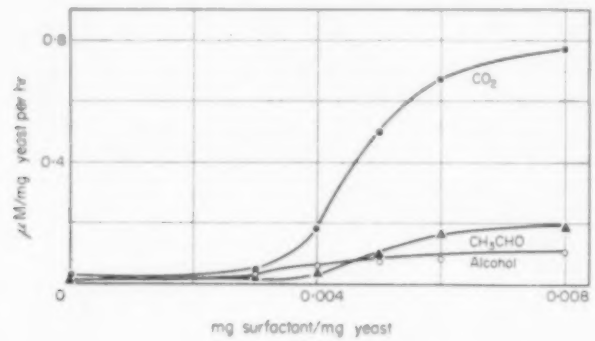

FIG. 5. Anaerobic decarboxylation of pyruvate vs. benzalkonium chloride concentration. Yeast, 10 mg/ml wet weight. Sodium pyruvate concentration was 0·05 M. Buffered as in Fig. 1. Nitrogen atmosphere. Average of two experiments.

the control suspensions was negligible. Above 0·004 surfactant concentration decarboxylation occurred at increasing rates. The concomitant appearances of acetaldehyde and alcohol and the failure to detect lactate indicated that the carboxylase enzyme was involved rather than the dismutation reaction occurring anaerobically in intact yeast below pH 4.[9]

In the aerobic experiments of Fig. 6 the same type of decarboxylation curve as seen in Fig. 5 was obtained. Below 0·004 surfactant, pyruvate was metabolized at the theoretical R.Q. of 1·2. The aerobic decarboxylation curve was obtained by assuming a

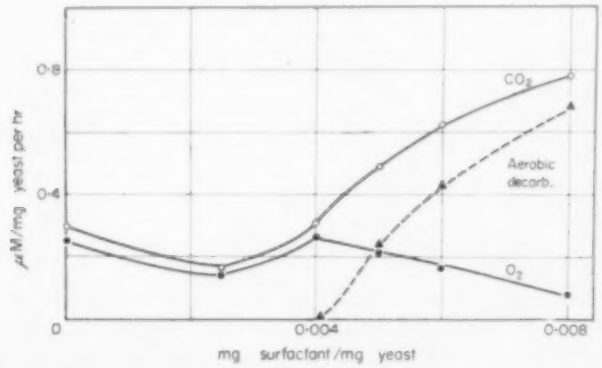

FIG. 6. Aerobic decarboxylation of pyruvate vs. benzalkonium chloride concentration. Conditions similar to those of Fig. 5, except air replaced nitrogen in flasks. Average of two experiments.

continued 1·2 value for R.Q. at the higher surfactant concentrations and subtracting 1·2 times the oxygen consumption from total CO_2 production at the corresponding surfactant concentration. In both the anaerobic and the aerobic experiments it was found that decarboxylation of pyruvate occurred in or on the cell, since the supernatant showed no ability to decarboxylate.

The correlation of the membrane effects with the metabolic effects in surfactant treated cells

In Fig. 7 the percentage inhibition of fermentation is plotted against percentage net K^+ loss. It can be seen that the anaerobic metabolism as measured by glucose utilization or CO_2 production showed results similar to the results of percentage staining vs. percentage K^+ loss obtained in Fig. 4. In addition, in Fig. 7 it is seen that the decarboxylation of pyruvate expressed as the percentage of the maximum decarboxylation rate was equal to the percentage K^+ loss for each surfactant concentration used.

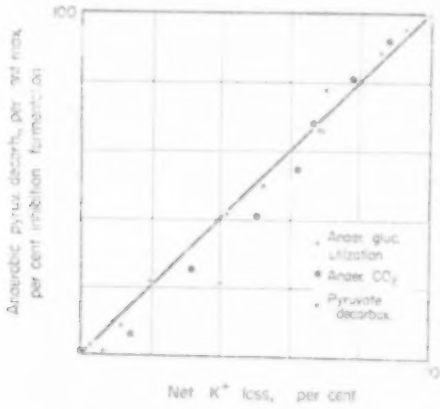

FIG. 7. Relationship of potassium loss to fermentation and anaerobic decarboxylation of pyruvate. Glucose concentration was 0·1 M for anaerobic CO_2, 0·03 M for anaerobic glucose utilization. Yeast, 5 mg/ml (wet weight), with glucose substrate, 10 mg/ml (wet weight), with pyruvate. All suspensions buffered as given in Fig. 1. Potassium loss corrected for control potassium loss. Benzalkonium chloride concentration: 0–16 mg/g yeast. Pyruvate and anaerobic CO_2 determinations by single experiments. Anaerobic glucose utilization, two experiments.

DISCUSSION

It is evident from the foregoing experiments that there is a high correlation between K^+ loss on the one hand and fermentation or decarboxylation of pyruvate on the other hand. As surfactant concentration is increased, greater numbers of cells simultaneously release all or nearly all of their K^+, cease to ferment glucose, and commence to decarboxylate pyruvate.

In an earlier paper from this laboratory[11] it was shown that low concentrations (below 0·004–0·005 mg surfactant/mg yeast) of benzalkonium ion stimulate oxygen consumption at pH 5·5 and inhibit the mechanism responsible for the Pasteur effect. At such low surfactant concentrations, membrane permeability as measured by net K^+ loss (Fig. 2) is not affected appreciably. The actions of benzalkonium chloride on yeast under the given conditions can thus be divided into two concentration zones.

Zone I: The prelytic zone below 0·004–0·005 (mg surfactant/mg yeast) surfactant concentration. In this zone oxygen consumption is increased and the Pasteur mechanism is inhibited,[11] but membrane permeability and fermentation are not appreciably altered. Zone II: Above 0·004–0·005 concentration all-or-none responses occur with regard to release of K^+, inhibition of fermentation, and decarboxylation of exogenous pyruvate. In this zone oxygen consumption also declines[11] but it has not been determined as yet whether this measure of metabolism follows an all-or-none pattern. In zone II it becomes evident that fermentation of glucose and decarboxylation of pyruvate are directly and inversely correlated, respectively, with the presence of an intact cell membrane. The inhibition of fermentation in zone II probably is not the result of direct inhibition of fermentation enzymes by surfactant, because direct studies on enzyme systems show that much higher concentrations of surfactants are needed to inhibit various enzymes than are needed to inhibit cellular metabolism.[1] It is more likely that benzalkonium ion either inhibits glucose transport to the fermentation sites or that benzalkonium causes loss from the cell (with consequent dilution) of necessary coenzymes or cofactors needed in fermentation. Perhaps both of these factors are responsible for inhibition of metabolism. However, alteration of the membrane does not necessarily cause release of fermentation enzymes themselves from the cell.[12]

The finding that pyruvate is decarboxylated when membrane permeability is affected is in agreement with previous findings.[10, 12] It may be suggested that the carboxylase enzyme is located in the periphery of the cell, protected from the external medium perhaps by the cell membrane alone. In the present studies, however, it was found that surfactant did not confer on the cells an ability to metabolize anaerobically glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, hexose diphosphate, or 3-phosphoglycerate. Such findings suggest that penetration of the cell membrane alone is not sufficient to bring about metabolism of an intermediate. The inability of surfactant-treated yeast to metabolize the above intermediates under anaerboic conditions lends support to the contention of Rothstein *et al.*[12] that the fermentation enzymes are in a structure or matrix which is not directly affected by an increase in membrane permeability. However, it is possible that loss of other cofactors for fermentation is more critical than loss of cocarboxylase needed for decarboxylation of pyruvate. Thus, failure to ferment phosphorylated intermediates could be accounted for on this basis.

If the postulated fermentation structures in the cell do exist, there are several possible explanations for the finding that pyruvate, but not the phosphorylated intermediates tested, are metabolized anaerobically when the membrane is no longer intact. One possibility is the inactivation by surfactant of one or more of the enzymes responsible for the ultimate conversion of 3-phosphoglycerate to pyruvate. In a preliminary experiment in this laboratory the anaerobic metabolism of glucose was inhibted nearly 100 per cent by treating yeast cells with benzalkonium chloride. The control suspension and the surfactant-treated suspension were then alternately frozen and thawed to break down further the cell structure. Metabolism in the control and surfactant-treated suspensions was then rechecked. The surfactant-treated preparation after freezing and thawing exhibited over 50 per cent of the activity of the frozen and thawed control preparation. Although such an experiment does not conclusively prove that inactivation of the enzymes is not involved in the action of surfactant, it does place such an explanation in doubt. Furthermore, if the fermentation structures are well-organized

for the best metabolic usage of glucose, it seems unlikely that the interior enzymes of the fermentation chain, but not hexokinase or carboxylase at either end of the chain, should be inhibited. Two other explanations are at hand: Either carboxylase is not a part of the fermentation structure, or a further barrier exists to the penetration of the fermentation structure by the phosphorylated intermediates. Experimental evidence is not at present available to allow a choice of one over the other.

Acknowledgement—The writer is grateful to Dr. Aser Rothstein of the University of Rochester for suggesting a staining technique for determining the membrane all-or-none response.

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Biochemical Pharmacology, 1960, Vol. 5, pp. 87–90. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECTS OF DRUGS ON BARBITAL ANAESTHESIA IN MICE

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(*Received* 12 *May* 1960)

Abstract—The effects of drugs on barbital induction time in mice have been examined: special attention was paid to compounds known to potentiate hexobarbital anaesthesia in this species. Brain barbital concentrations at onset of anaesthesia were recorded after pre-treatment with reserpine, 5-hydroxytryptamine, chlorpromazine or benactyzine. All four drugs reduce the induction time of barbital anaesthesia without increasing its rate of brain penetration. Possible mechanisms of action are discussed. It is suggested that the criterion of reduced induction for barbital anaesthesia should not be regarded as necessarily indicating an increased permeability of the blood–brain barrier.

INTRODUCTION

BUTLER[1] has shown that the delay in onset of anaesthesia after an intravenous injection of barbital results from the hypnotic's slow rate of penetration into the brain. Prior administration of physostigmine halves this induction time by increasing the permeability of the blood–brain barrier to the barbiturate.[2] Other workers have since claimed that certain enzymes, notably trypsin, chymotrypsin and hyaluronidase,[3] or adrenalectomy,[4, 5] can shorten the induction time of barbital anaesthesia by altering the permeability of the blood–brain barrier. We therefore attempted to determine whether other drugs that potentiate barbiturates also affect the blood–brain barrier.

METHODS

Male fawn mice (GFF strain, body weight range 16—22 g) were used.

The induction time for barbital was measured by the method of Greig and Mayberry.[2]

Groups of ten mice were injected intravenously with sodium barbital (5·5 mg in 0·4 ml/20 g), each injection taking 20—30 sec. The interval between the injection and the loss of the righting reflex was taken as the induction time. The experiments were conducted at room temperature (approx. 19 °C), and groups of mice given barbital only were always included, to allow for day-to-day variations in response.

Brain barbital concentrations were determined by Butler's method.[1] Groups of four mice were injected intravenously with sodium barbital (5·5 mg in 0·4 ml/20 g) and then killed after intervals of 2, 5, 10, 15, 20, 25, 30 and 35 min. The brains from each group were weighed together and assayed for total barbital.

RESULTS

The changes in barbital induction time produced by pre-treating the mice with drugs known to increase the duration of hexobarbital hypnosis in mice are given in Table 1; the results in Table 2 refer to a miscellany of pharmacological agents.

TABLE 1. CHANGES IN INDUCTION TIME OF BARBITAL ANAESTHESIA PRODUCED BY DRUGS POTENTIATING HEXOBARBITAL HYPNOSIS

Drug	Dose (mg/kg)	Route	Time before barbital (min)	Group mean induction time (min. ± s.e.)*	
				Test	Control
5-Hydroxy-tryptamine	2·5	intraperitoneal	10	12·7 ± 0·8	18·3 ± 0·6
	5·0			10·0 ± 0·6	
	20·0			7·7 ± 0·4	
Reserpine	0·5	intraperitoneal	60	12·1 ± 0·7	17·8 ± 0·9
	2·0			8·0 ± 0·4	
	5·0			5·2 ± 0·4	
Chlorpromazine	0·5	intraperitoneal	5	11·3 ± 0·8	18·1 ± 1·0
	1·0			9·8 ± 0·8	
	2·0			7·0 ± 0·6	
Benactyzine	2·5	intraperitoneal	30	11·9 ± 0·7	17·7 ± 0·8
	10·0			10·5 ± 0·7	
SKF525A	50	oral	40	17·3 ± 0·9	17·6 ± 0·9
	100			18·1 ± 0·9	
Phenergan	2·5	intraperitoneal	15	14·1 ± 0·8	18·4 ± 0·9
	5·0			11·1 ± 0·7	
Mepyramine	2·5	intraperitoneal	15	16·8 ± 1·0	18·5 ± 0·8
	5·0			13·2 ± 0·8	
	10·0			10·5 ± 0·3	

* Ten animals per group.

TABLE 2. CHANGES IN INDUCTION TIME OF BARBITAL ANAESTHESIA PRODUCED BY VARIOUS DRUGS INTRAPERITONEALLY

Drug	Dose (mg/kg)	Time before barbital (min)	Group mean induction time (min ± s.e.)*	
			Test	Control
Eserine	0·4	Immediate	21·5 ± 0·6	18·4 ± 0·6
	0·4	30	11·6 ± 0·6	
Amphetamine	1·0	15	18·2 ± 0·7	16·6 ± 0·6
	2·5		20·2 ± 0·9	
	5·0		27·2 ± 1·0	
	10·0		31·4 ± 1·1	
Metrazole	10·0	15	19·6 ± 1·0	18·6 ± 0·6
	20·0		18·2 ± 0·7	
Nikethamide	10·0	15	17·0 ± 0·8	17·7 ± 0·6
	20·0		15·3 ± 0·8	
Picrotoxin	1·0	15	17·8 ± 0·9	18·6 ± 0·9
Atropine	2·5	15	16·1 ± 0·9	17·4 ± 0·5
	5·0		15·0 ± 0·9	
Lysergic acid diethylamide	1·0	5	18·0 ± 0·5	19·8 ± 0·6
	5·0	5	17·0 ± 0·9	
	1·0	60	19·4 ± 1·0	
	5·0	60	15·5 ± 0·9	
Pilocarpine	1·0	15	15·5 ± 0·4	16·9 ± 0·6

* Ten animals per group.

Induction time was significantly reduced ($P < 0.001$) by pre-treatment with reserpine, 5-hydroxytryptamine, chlorpromazine or benactyzine, but was unaffected by SKF.525-A (β-diethylaminoethyldiphenylpropylacetate). The decreases in induction time were not associated with increased rates of penetration into the brain by the barbital. In fact the brain barbital concentrations at the onset of hypnosis were significantly less than that found for control animals (Table 3). The results obtained

Table 3. Brain concentration of barbital after pre-treatment with drugs

Drug	Dose (mg/kg) intraperitoneal	Time before barbital (min)	Group mean induction time (min ± s.e.)*	Brain barbital concentration at induction (moles × 10^{-6}/g wet tissue)
Nil	—	—	17·9 ± 0·7	1·12
5-Hydroxytryptamine	20·0	10	7·7 ± 0·4	0·68
Reserpine	2·0	60	8·0 ± 0·4	0·65
Chlorpromazine	2·0	5	7·0 ± 0·6	0·79
Benactyzine	2·5	30	11·9 ± 0·7	0·89

* Ten mice per group.

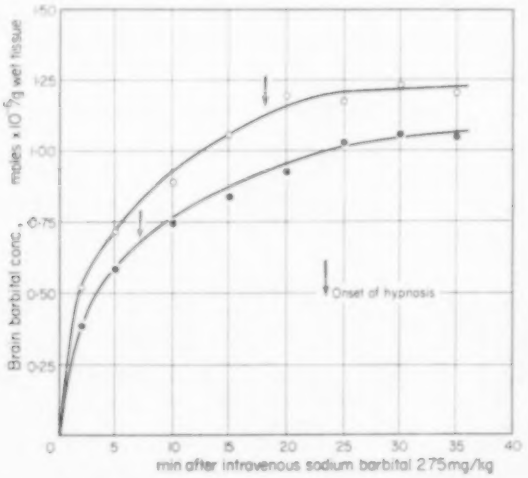

Fig. 1. The effect of reserpine on the rate of penetration of sodium barbital into mouse brain. ○——○ barbital alone; ●——● barbital 60 min after intraperitoneal reserpine 2·0 mg/kg.

with reserpine are illustrated in Fig. 1. The decrease in induction time produced by 5-hydroxytryptamine was partially reversed by pre-treatment with lysergic acid diethylamide 10 mg/kg i.p. (Table 4).

Certain analeptics—metrazole, nikethamide and picrotoxin—did not affect barbital induction time in small doses, but amphetamine (5 or 10 mg/kg) markedly increased it.

TABLE 4. MODIFICATION BY LYSERGIC ACID DIETHYLAMIDE OF THE REDUCTION IN BARBITAL INDUCTION TIME PRODUCED BY SEROTONIN

Group mean induction time — min ± s.e.*		
Control	10 min after after 5-hydroxytryptamine 20 mg/kg intraperitoneally	10 min after 5-hydroxytryptamine 20 mg/kg intraperitoneally and 1 hr after lysergic acid diethylamide 10 mg/kg intraperitoneally
17·8 ± 0·6	7·6 ± 0·6	13·2 ± 0·9

* Ten mice per group.

Of the other drugs tested, only the anti-histamines, phenergan and mepyramine reduced the induction time.

DISCUSSION

The results summarized above showed that pre-treatment of mice with reserpine, 5-hydroxytryptamine, benactyzine, chlorpromazine, phenergan or mepyramine reduced the delay in onset of barbital anaesthesia.

With reserpine, 5-hydroxytryptamine, benactyzine and chlorpromazine, the reductions were not the result of increased rate of penetration into the brain by the barbital, because the brain barbital concentrations at the onset of anaesthesia were less than normal. The reduced induction times might have resulted from increased sensitivity of the brain cells to barbital or have been reflections of the hypothermia produced by these drugs.[6–8]

The experiments recorded here showed that a decrease in the induction time of barbital anaesthesia did not necessarily imply an increased permeability of the blood–brain barrier.

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Biochemical Pharmacology, 1960, Vol. 5, pp. 91–95. Pergamon Press Ltd., Printed in Great Britain.

COMPARATIVE EFFECTS OF MONOAMINE OXIDASE INHIBITORS ON MONOAMINE OXIDASE AND DIAMINE OXIDASE*

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(*Received* 13 *May* 1960)

Abstract—The effect of several monoamine oxidase (MAO) inhibitors and related compounds on guinea pig liver MAO and diamine oxidase (DAO) was examined *in vitro* and after administration to animals. The results showed that the MAO inhibitors possessing the hydrazine moiety inhibited both MAO and DAO, while two phenyl*cyclo*propylamine MAO inhibitors did not affect DAO. Hydrazine derivatives which did not block MAO retained a DAO inhibitory action. After administration of iproniazid to rats, DAO was inhibited long after the reported time for the drug to disappear from tissues; this finding suggests that the action of iproniazid on DAO, as well as on MAO, is "irreversible".

THE question of the mechanisms involved in the diverse clinical effects induced by monoamine oxidase (MAO) inhibitors is an intriguing one. Included among the clinical effects are orthostatic hypotension, abatement of anginal pain and an antidepressant effect.[1] In laboratory animals a degree of central stimulation, anticonvulsant and sympathomimetic effects have been observed.[1]

The diversity of the pharmacologic effects emphasizes the desirability of a more complete knowledge of the actions, other than inhibition of MAO, which might be induced by these drugs.

In the present studies we have investigated the effects on diamine oxidase (DAO) *in vivo* and *in vitro* of a variety of drugs (Fig. 1) including: (a) MAO inhibitors containing the hydrazine moiety, (b) non-hydrazine MAO inhibitors, and (c) hydrazine derivatives which are not MAO inhibitors.† The experiments demonstrate that the hydrazine moiety confers DAO blocking activity, whether or not the molecule also blocks MAO activity, while the non-hydrazine MAO inhibitors tested do not block DAO. The studies further suggest that the action *in vivo* of iproniazid on DAO as well as MAO is "irreversible".

METHODS

Histamine was estimated fluorometrically by the method of Shore *et al.*[2] and serotonin by the method of Bogdanski *et al.*[3]

DAO activity was estimated by measurement of the rate of disappearance of histamine added to tissue homogenates, while MAO activity was measured in a similar fashion by following the disappearance of serotonin.

* A preliminary report of these findings was presented before a meeting of the American Society for Pharmacology and Experimental Therapeutics, Chicago, April, 1960.

† Iproniazid, isoniazid, Ro 4-1634 and Ro 4-1038 were kindly supplied by Hoffmann-La Roche, Inc., and SKF 385 and SKF 556 by Smith, Kline and French Laboratories.

Tissues were homogenized in 9 vols. of 0·2 M phosphate buffer, pH 7·2. After pre-incubation in a Dubnoff metabolic shaker for 15 min at 37 °C in an atmosphere of air, histamine or serotonin was added to make a final concentration of 7·5 μg/ml histamine or 12·5 μg/ml serotonin. Samples were removed 20 min later for serotonin assay or 60 min later for histamine assay.

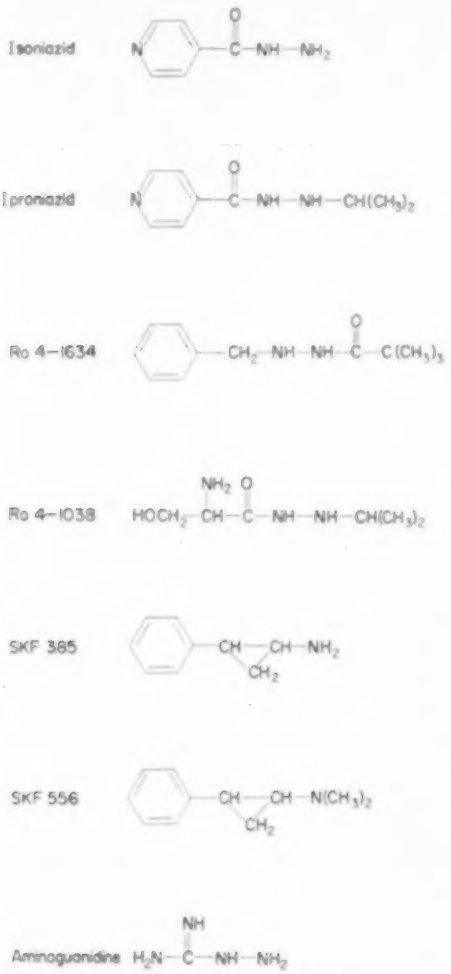

FIG. 1. Monoamine oxidase inhibitors

Inhibition of the enzymes *in vitro* was carried out by addition of inhibitors to the homogenates at the beginning of the 15-min pre-incubation period. Homogenates to which no inhibitor was added served as controls. The extent of inhibition *in vivo* after intraperitoneal administration of the inhibitors was measured in a similar manner; tissue homogenates from untreated animals served as controls.

RESULTS

Inhibition of DAO and MAO in vitro

Guinea pig liver was used in these experiments since it is a tissue rich in both MAO and DAO. The extent of inhibition of DAO and MAO activities by various concentrations of the drugs is shown in Table 1. A marked inhibition of DAO was obtained

TABLE 1. INHIBITION OF GUINEA PIG LIVER MAO AND DAO *in vitro*

Inhibitor	Molar Concentration of Inhibitor							
	10^{-3}		10^{-4}		10^{-5}		10^{-6}	
	MAO	DAO	MAO	DAO	MAO	DAO	MAO	DAO
Iproniazid	98	75	11	68	0	16	0	10
Ro 4-1634	100	100	100	100	100	100	0	27
Ro 4-1038	100	100	78	86	3	39	0	9
SKF 385	100	13	100	0	95	0	0	0
SKF 556	88	15	87	4	79	0	1	0
Aminoguanidine	0	100	0	100	0	100	0	100
Isoniazid	2	93	2	60	0	14	0	6

Experiments were performed as described under "Methods". Each value represents the average percentage inhibition in from three to six experiments.

with all of the hydrazine derivatives, but not with the phenyl*cyclo*propylamines. Conversely, a marked inhibition of MAO was obtained with the phenyl*cyclo*propylamines and certain of the hydrazine derivatives. Aminoguanidine and isoniazid did not inhibit MAO even at high concentrations.

Inhibition of DAO and MAO in vivo

Measurement of DAO and MAO activity of liver from guinea pigs treated with the various drugs revealed a picture of inhibition (Table 2) very similar to that seen in the studies *in vitro*. All of the hydrazine derivatives caused a marked inhibition of DAO.

TABLE 2. INHIBITION OF GUINEA PIG LIVER MAO AND DAO *in vivo*

Inhibitor	1 hr		24 hr	
	MAO	DAO	MAO	DAO
Iproniazid (100 mg/kg)	100	100	100	94
Ro 4-1634 (100 mg/kg)	100	100	100	46
Ro 4-1038 (100 mg/kg)	100	100	100	71
SKF 385 (3 mg/kg)	100	0	6	0
SKF 556 (20 mg/kg)	62	0	14	0
Aminoguanidine (20 mg/kg)	0	91	0	78
Isoniazid (100 mg/kg)	0	70	0	80

Experiments were performed as described under "Methods". Each value represents the average percentage inhibition in from three to six experiments.

but only certain of these compounds caused an inhibition of MAO. The phenycyclopropylamines were highly active in blocking MAO, but were devoid of DAO blocking activity.

After administration of 100 or 195 mg of iproniazid per kg, to rats, the activity of DAO in the small intestine, the major source of DAO in this species, was strongly inhibited for at least 48–72 hr (Table 3).

TABLE 3. INHIBITION OF RAT SMALL INTESTINE DAO *in vivo* BY IPRONIAZID

Time after iproniazid (hr)	Dose of iproniazid	
	100 mg/kg	195 mg/kg
18	92	—
24	77	85
48	59	67
72	57	—

Experiments were performed as described under "Methods". Each value represents the average percentage inhibition in from three to six experiments.

DISCUSSION

It is possible to draw certain conclusions from the experiments with the representative compounds described in this paper.

Hess *et al.*[4], showed that 24 hr after administration of iproniazid, 195 mg/kg, to rats, the drug had essentially disappeared from the tissues, but that inhibition of MAO persisted much longer. These results constituted one of the means of demonstration of the "irreversible" nature of iproniazid-induced inhibition of MAO. The present results demonstrate that iproniazid, which has been reported previously to block DAO *in vitro*,[5] inhibited DAO *in vivo* for at least 48 hr after the same dose used by Hess *et al.*[4] After only one-half this dose (100 mg/kg), inhibition of DAO activity again persisted for at least 48–72 hr. The results are suggestive that DAO as well as MAO is "irreversibly" inhibited by iproniazid.

The data also demonstrate that not only iproniazid, but also other MAO inhibitors which contain the hydrazine moiety, can block DAO as well as MAO, *in vitro* and *in vivo*. Indeed, DAO was blocked *in vitro* more strongly than MAO by certain concentrations of the MAO inhibitors, iproniazid, Ro 4-1038, and Ro 4-1634. It is noteworthy that the hydrazine group need not be in the free or "carbonyl reagent" form for blockade of DAO, as might be suspected from reports of inhibition of DAO by carbonyl reagents such as semicarbazide.[6] The present results suggest that inhibition of DAO by hydrazine containing molecules does not necessarily proceed by reaction with a carbonyl group.

The absence of DAO inhibition at high concentrations of the potent MAO inhibitors SKF 385 and SKF 556 demonstrates that DAO inhibition is not necessarily a corollary of MAO inhibition.

A further separation of enzyme inhibitory activity is obtained with isoniazid and with aminoguanidine, compounds which long have been known to inhibit DAO.[5, 7]

The inability of guinea pig liver to metabolize histamine *in vitro* in the presence of aminoguanidine or isoniazid (Table 1), and the unaltered ability to destroy histamine

during complete blockade of MAO activity by the addition of SKF 385 or SKF 556 (Table 1), suggest that guinea pig liver MAO is incapable of metabolizing histamine. Similar results were obtained when a homogenate of beef liver was substituted for guinea pig liver. The results differ from those of others who used very high substrate concentrations (10^{-2} M) and on the basis of manometric measurements reported that beef liver MAO could metabolize histamine.[8]

It is of interest that although some of the hydrazine-containing MAO inhibitors (Ro 4-1634 and Ro 4-1038) inhibit guinea pig liver DAO more strongly than MAO at low concentrations *in vitro*, the action of these drugs *in vivo* appears to be more rapidly reversed in the case of DAO than MAO, suggesting either a different mode of action of the inhibitors or a difference in the rates of enzyme synthesis.

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Biochemical Pharmacology, 1960, Vol. 5, pp. 96–102. Pergamon Press Ltd., Printed in Great Britain.

STUDIES ON THE MODE OF ACTION OF PSICOFURANINE*

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(*Received* 29 *April* 1960; *revised* 23 *May* 1960)

Abstract—The mode of action of the antibiotic psicofuranine was studied using *E. coli B*. The inhibition of bacterial growth by psicofuranine was only transitory and the cells were able to overcome it after an extended lag phase. The inhibitory action of psicofuranine could be reversed by guanine and its derivatives; other purines and pyrimidines were inactive as reversing agents. Cells growing in the presence of psicofuranine excreted xanthosine into the medium. The measurements of purine synthesis from glycine-1-^{14}C in whole cells under the influence of psicofuranine showed a decreased isotope incorporation into guanine with a simultaneous increase in the radioactivity of xanthine. No effect on the incorporation of glycine-1-^{14}C into adenine was observed. The same results were obtained when the conversion of hypoxanthine-8-^{14}C into adenine and guanine as measured in inhibited cells. In the presence of psicofuranine, conversion of xanthine-8-^{14}C into both guanine and adenine was inhibited. These results showed that psicofuranine inhibited the conversion of xanthosine-5′-phosphate to guanosine-5′-phosphate.

PSICOFURANINE was isolated from culture media of *Streptomyces hygroscopicus var. decoyinine*[1] and found to possess antibacterial[2] and antitumor[3] properties. Its structure was established as 6-amino-9-D-psicofuranosylpurine[4] and it may, therefore, be visualized as a nucleoside analogue, containing the anomalous hexose, psicose, instead of the normally occurring pentose. Hanka[5] found that the inhibitory effect of psicofuranine on growth of *S. aureus* could be reversed by guanine, guanosine and guanosine-5′-phosphate† at concentrations of from 5 to 10 μg/ml. Other purines and pyrimidines showed some reversal effect at much higher levels. These results led Hanka[5] to the suggestion that psicofuranine interfered with the synthesis of G5′P from X5′P.

This communication presents conclusive evidence that in *E. coli B*, psicofuranine inhibits the conversion of X5′P to G5′P.

METHODS

Cultures of *E. coli B* were maintained on Difco Nutrient Agar slopes. Bacteria were grown in the minimal salts-glucose medium, described by Davis and Mingioli.[6] In the growth experiments, 4 ml of sterile medium were placed into sterile colorimeter tubes and each tube was inoculated with 1–2 × 10^7 cells from a liquid medium culture

* 6-Amino-9-D-psicofuranosylpurine.

† The following abbreviations are used: G5′P, guanosine-5′-phosphate; X5′P, xanthosine-5′-phosphate; I5′P, inosine-5′-phosphate; A5′P, adenosine-5-phosphate; ATP, adenosine-triphosphate; GTP, guanosinetriphosphate.

grown for 12 hr. The tubes were incubated at 37 °C and growth was followed by measuring the light transmittance at 520 mμ in a Bausch and Lomb colorimeter. Viable cell counts were made by plating $1 : 10^6$ and $1 : 10^7$ dilutions of the cell suspensions three times each and counting the colonies after 24 hr of incubation.

Xanthosine, which was excreted into the medium by inhibited cells, was isolated and identified in the following manner: After removal of cells by centrifugation, all ultraviolet light absorbing material from 1000 ml of growth medium was adsorbed on 3 g of activated charcoal (Darco G-60) and eluted with a mixture of 65% water, 25% ethanol and 10% concentrated ammonium hydroxide. Five elutions, 150 ml each, were made to recover 98 per cent of the ultraviolet light absorbing material from the charcoal. The combined eluates, after filtration through Celite, were evaporated to dryness in a rotary flash evaporator at 35 °C and the dry crystals were dissolved in 10 ml of water containing 0·25 ml of concentrated ammonium hydroxide. The solution was poured onto a column of Dowex-1 (chloride form, 8·3 cm × 1 cm) and the resin was eluted with 0·1 M NH_4Cl, adjusted to pH 9·5 with ammonium hydroxide.[7] The eluate was collected in 10 ml fractions and analyzed for absorption at 260 mμ. Separation of the components in the eluate from charcoal was also achieved by paper chromatography, using a *n*-butanol–ammonia solvent system.[8] For the paper rechromatography of the isolated xanthosine and its separation from xanthine, the *iso*amyl alcohol–Na_2HPO_4 mixture of Carter[9] was used. Spots of xanthosine were detected by examining the paper chromatograms in ultraviolet light and by spraying with a solution of sodium metaperiodate and Schiff's reagent.[10] Free pentose, resulting from hydrolysis of xanthosine with 1 N HCl for 30 min in boiling water bath, was detected on paper chromatograms by its reaction with the anilinephthalate reagent of Partridge.[11]

Experiments with radioactive tracers were made by techniques similar to those of Tomisek *et al.*[12, 13] Two Erlenmeyer flasks containing 20 ml of washed cell suspension in the growth medium (transmittance 20 per cent) were incubated for 30 min at 37 °C. Two milligrams of psicofuranine in 1 ml of water were added to one flask. The control flask received 1 ml of water. The incubation was continued for 10 min, after which radioactive substrates were added to both flasks. The cells were allowed to assimilate the added radioactive compounds for 10 min and their metabolism was stopped by pouring the cell suspension onto 10 g of ground ice in a 50 ml centrifuge tube. Cells were sedimented by centrifugation at 0 °C and washed twice with 2 ml of ice-cold physiological saline. The sedimented and washed cells were then hydrolyzed for 60 min in 3 ml of 1 N HCl in a boiling water bath. This treatment liberated the purine bases from nucleic acids and nucleotides. The resulting suspension was centrifuged at 10,000 × *g* for 10 min and the sedimented residual proteins were washed twice with 1 ml of water and dried *in vacuo*. The washings were combined with the original 1 N HCl extract and dried *in vacuo* over NaOH. The dry residues were dissolved in 0·2 ml of water. Separation of adenine, guanine, hypoxanthine and xanthine in this solution was achieved by two-dimensional paper chromatography,[14] using *iso*propanol-HCl[15] as the first and *n*-butanol-NH_3[8] as the second solvent system. Since the bacteria in the amount used did not contain enough hypoxanthine and xanthine to permit their detection by ultraviolet light absorption, carrier hypoxanthine and xanthine were applied on the chromatograms prior to the extracts. Purines were eluted from the paper with 5 ml of 0·1 N HCl and their absorption spectra determined in a

recording spectrophotometer (Cary, model 11). Aliquots of the eluates were taken for determination of radioactivity. Owing to the short incubation period, no spectrophotometrically demonstrable increase in total adenine and guanine concentration was observed after quantitative elution of the respective spots. Therefore, the radioactivity measurements were calculated as counts per minute in each purine and comparison was made by expressing the counts in each individual purine as percentage of the total radioactivity in the four purine bases. Specific activities of adenine and guanine were calculated after quantitative elution of the respective spots on paper chromatograms with 0·1 N HCl and measurements of ultraviolet light absorption at 260 mμ for adenine and at 250 mμ for guanine. The extinction coefficients used were those given by Wyatt.[14] Location of the spots on the paper chromatograms was also detected by radioautography. Developed chromatograms were left in contact with Kodak no-screen X-ray film for a period of from 2 to 3 weeks.

Radioactive carbon (^{14}C) was measured in a Packard tri-carb liquid scintillation spectrometer. The counting solution was a mixture of toluene and absolute ethanol (7 : 3) with 0·4% 2 : 5-diphenyloxazole and 0·01% 1 : 4-di-[2-(5-phenyloxazolyl)] benzene as the phosphors. Protein was determined by the optical method of Warburg and Christian.[16]

Glycine-1-^{14}C (14·7 μc/mg), hypoxanthine-8-^{14}C (5·4 μc/mg) and xanthine-8-^{14}C (1·6 μc/mg) were purchased from Volk Radiochemical Company and used without dilution. Adenine, guanine, hypoxanthine, xanthine, guanosine and xanthosine were products of Nutritional Biochemical Corporation. The other purine and pyrimidine derivatives were purchased from California Corporation for Biochemical Research. Xanthosine-5′-phosphate was synthesized from guanosine-5′-phosphate by diazotation and hydrolysis of the diazonium compound.[17, 18]

RESULTS

Growth curves of *E. coli B* in the presence of increasing concentration of psicofuranine are shown in Fig. 1. It may be seen that with increasing concentrations of

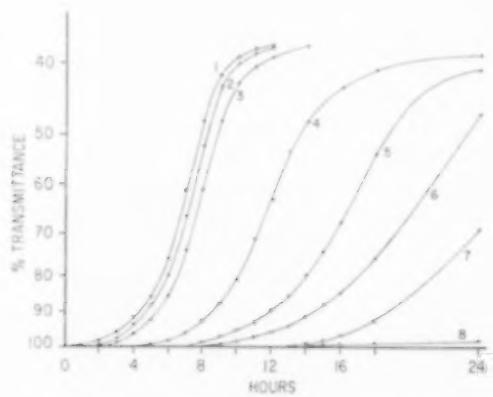

FIG. 1. Growth curves of *E. coli B* in the presence of increasing concentrations of psicofuranine. Concentrations of psicofuranine per ml: 1 = 0; 2 = 5 μg; 3 = 10μg; 4 = 50 μg; 5 = 75 μg; 6 = 100 μg; 7 = 150 μg; 8 = 200 μg.

psicofuranine a longer lag phase occurred before the onset of growth. With relatively low concentrations of the antibiotic (5–10 μg/ml) the cells eventually overcame the inhibition completely and grew at the same rate as the control cells. With higher concentrations of psicofuranine, the growth rate during the logarithmic phase was inhibited, the extent of inhibition being dependent on the concentration of the inhibitor. When psicofuranine was added to a growing culture of *E. coli B* at the beginning of the logarithmic phase in the concentration 100 μg/ml, which was inhibitory to the inoculum for at least 7 hr (Fig. 1), it produced only a partial inhibition of growth, as shown in Fig. 2. Microscopic examination of cultures growing in the

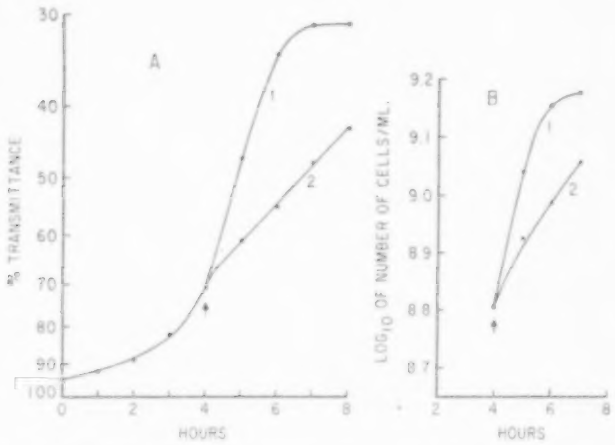

FIG. 2. Effect of psicofuranine on bacterial growth when added at the beginning of the logarithmic phase of growth, measured by light transmittance of the cell suspension and by viable cell counts. Two test tubes with 4 ml of medium were inoculated with 2×10^7 cells and at the time indicated by the arrow, psicofuranine (100 μg/ml) was added to one test tube. From this point on, samples were taken for the viable cell count determination. A = light transmittance measurements; B = viable cell counts; curve 1 = without psicofuranine; curve 2 = with added psicofuranine.

presence of psicofuranine failed to reveal any elongated or filamentous forms of the cells. Viable cell counts, determined in the experiment shown in Fig. 2, confirmed that light transmittance measurements were indeed measurements of the growth of the bacterial population.

Attempts to prevent the inhibitory effect of psicofuranine on the growth of *E. coli B* by purines, pyrimidines, and their derivatives are summarized in Table 1. The data show the striking superiority of guanine and related compounds over the other substances in their ability to abolish the effect of psicofuranine. Results of a detailed reversal study with guanine, present in the medium in the concentration range from 0 to 10 μg/ml, are shown in Fig. 3. Similar results were obtained with guanosine and G5′P as reversing agents.

Excretion of an ultraviolet light absorbing substance into the medium by cells growing in the presence of psicofuranine was noticed when the absorption spectra of the growth medium with 100 μg/ml of the psicofuranine were determined at zero time and after 24 hr of incubation; at this time the bacterial growth was identical with the maximal growth of the control. Both spectra are reproduced in Fig. 4. Subtracting

TABLE 1. PREVENTION OF THE INHIBITORY ACTION OF PSICOFURANINE (100 μg/ml) ON THE GROWTH OF *E. coli B* BY PURINES AND PYRIMIDINES PRESENT IN THE MEDIUM
(The growth was measured after 8 hr.)

Reversing compound (10 μg/ml)	Growth in % of the control
Adenine	14·5
Guanine	102
Hypoxanthine	2
Xanthine	4
Cytosine	4
Uracil	2
Thymine	4
Adenosine	4
Guanosine	100
Inosine	4
Xanthosine	4
Cytidine	1
Uridine	1
Adenosine-5′-phosphate	6
Guanosine-5′-phosphate	102
Cytidine-5′-phosphate	2
Uridine-5′-phosphate	2
Deoxyadenosine	4
Deoxyguanosine	75
Deoxycytidine	4
Thymidine	2
Deoxyadenosine-5′-phosphate	4
Deoxyguanosine-5′-phosphate	62·5
Deoxycytidine-5′-phosphate	4
Thymidine-5′-phosphate	2
No additions	2

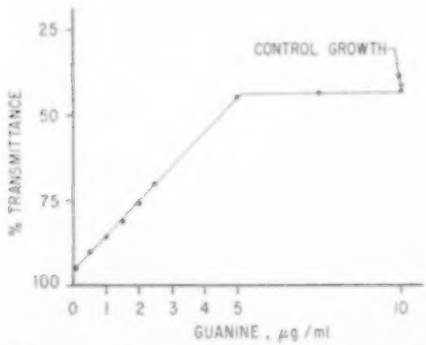

FIG. 3. Prevention of the inhibitory action of psicofuranine on growth of *E. coli B* by guanine. Concentration of psicofuranine was 100 μg/ml. The concentrations of guanine in the medium ranged from 0 to 10 μg/ml. Growth was measured after 8 hr.

the original spectrum at zero time from the final one after 24 hr, a difference spectrum was obtained which closely resembled that of xanthosine. No ultraviolet absorbing materials were excreted into the growth medium by the control cells. Isolation and identification of xanthosine was made from 1000 ml of growth medium of an inhibited

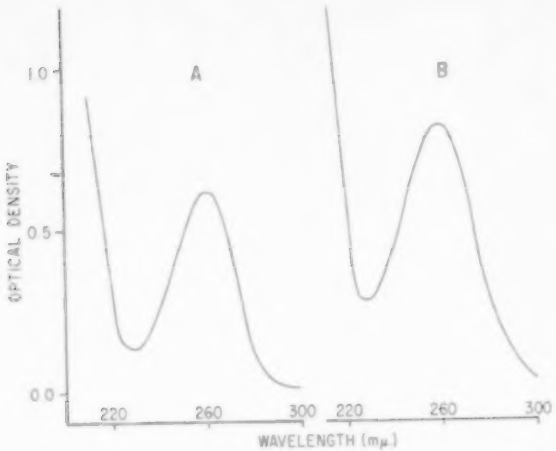

FIG. 4. Accumulation of an ultraviolet light absorbing material in the growth medium by cells growing in the presence of psicofuranine. A, ultraviolet light absorption spectrum of the medium with 100 μg of psicofuranine per ml at the beginning of the growth experiment (0 hr); B, ultraviolet light absorption spectrum of the medium after 24 hr of incubation. Media were diluted 1 : 10 with H_2O.

culture by charcoal adsorption and column chromatography of the charcoal eluate on Dowex-1. The elution pattern from the column is shown in Fig. 5. Component *A* was identified as psicofuranine and component *B* as xanthosine. Paper chromatography of the charcoal eluate in the *n*-butanol–NH_3 solvent system revealed only two spots: that of psicofuranine with $R_f = 0{\cdot}18$ and that of xanthosine with $R_f = 0{\cdot}02$. Identity of xanthosine was established by the following criteria: ultraviolet light absorption spectrum in 0·001 N HCl and in 1 N NaOH; paper chromatography in the *iso*amyl alcohol–Na_2HPO_4 system; hydrolysis with 1 N HCl and identification of resulting xanthine and ribose by paper chromatography. A control sample of xanthosine was

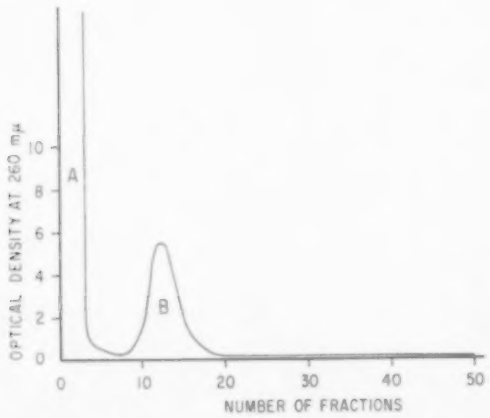

FIG. 5. Separation of psicofuranine and xanthosine on Dowex-1. The column was eluted with 0·1 *M* NH_4Cl (pH adjusted to 9·5 with NH_4OH). Peak A = psicofuranine; peak B = xanthosine.

TABLE 2. EFFECT OF PSICOFURANINE ON THE INCORPORATION OF GLYCINE-1-^{14}C INTO PURINE BASES OF *E. coli B*

Two Erlenmeyer flasks, each containing 20 ml of bacterial suspension (transmittance 20 per cent), were incubated for 30 min; then psicofuranine was added to one flask in the final concentration of 100 μg/ml. After 10 min, 5μc of glycine-1-^{14}C were added to both flasks and the incubation was continued for an additional 10 min. Washed cells were hydrolyzed in 1 N HCl in a boiling water bath and the purines isolated by two-dimensional paper chromatography.

	Without psicofuranine			With psicofuranine		
	Counts/min	% of the total purine radioactivity	Specific activity	Counts/min	% of the total purine radioactivity	Specific activity
Total purines	$3{\cdot}455 \times 10^5$	100	—	$2{\cdot}375 \times 10^5$	100	—
Adenine	$1{\cdot}535 \times 10^5$	44·5	$6{\cdot}20 \times 10^4$ counts/min per μM	$1{\cdot}090 \times 10^5$	46	$4{\cdot}40 \times 10^4$ counts/min per μM
Guanine	$1{\cdot}745 \times 10^5$	50·5	$6{\cdot}48 \times 10^4$ counts/min per μM	$0{\cdot}603 \times 10^5$	25·4	$2{\cdot}23 \times 10^4$ counts/min per μM
Hypoxanthine	$0{\cdot}0765 \times 10^5$	2·2	—	$0{\cdot}066 \times 10^5$	2·7	—
Xanthine	$0{\cdot}0985 \times 10^5$	2·8	—	$0{\cdot}616 \times 10^5$	26	—
Protein	—	—	$6{\cdot}44 \times 10^4$ counts/min per mg	—	—	$6{\cdot}03 \times 10^4$ counts/min per mg

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TABLE 3. EFFECT OF PSICOFURANINE (100 μg/ml) ON THE CONVERSION OF HYPOXANTHINE-8-^{14}C INTO OTHER PURINE BASES IN *E. coli B*; HYPOXANTHINE-8-^{14}C (5 μC) WAS ADDED TO EACH FLASK

(For experimental details, see legend to Table 2.)

	Without psicofuranine			With psicofuranine		
	Counts/min	% of the total purine radioactivity	Specific activity	Counts/min	% of the total purine radioactivity	Specific activity
Total purines	$3{\cdot}58 \times 10^5$	100	—	$2{\cdot}01 \times 10^5$	100	—
Adenine	$1{\cdot}66 \times 10^5$	46·4	$6{\cdot}64 \times 10^4$ counts/min per μM	$0{\cdot}97 \times 10^5$	48·3	$3{\cdot}88 \times 10^4$ counts/min per μM
Guanine	$1{\cdot}47 \times 10^5$	41·2	$5{\cdot}47 \times 10^4$ counts/min per μM	$0{\cdot}525 \times 10^5$	25·1	$1{\cdot}61 \times 10^4$ counts/min per μM
Hypoxanthine	$0{\cdot}113 \times 10^5$	3·2	—	$0{\cdot}095 \times 10^5$	4·7	—
Xanthine	$0{\cdot}326 \times 10^5$	9·1	—	$0{\cdot}420 \times 10^5$	20·9	—

TABLE 4. EFFECT OF PSICOFURANINE (100 μg/ml) ON THE CONVERSION OF XANTHINE-8-^{14}C INTO OTHER PURINE BASES IN *E. Coli B*; XANTHINE-8-^{14}C (2·5 μC) WAS ADDED TO EACH FLASK

(For experimental details, see legend to Table 2.)

	Without psicofuranine			With psicofuranine		
	Counts per min	% of the total purine radioactivity	Specific activity	Counts per min	% of the total purine radioactivity	Specific activity
Total purines	$10{\cdot}65 \times 10^4$	100	—	$6{\cdot}07 \times 10^4$	100	—
Adenine	$4{\cdot}00 \times 10^4$	37·6	$2{\cdot}99 \times 10^4$ counts/min per μM	$1{\cdot}12 \times 10^4$	18·5	$0{\cdot}864 \times 10^4$ counts/min per μM
Guanine	$5{\cdot}35 \times 10^4$	50·2	$3{\cdot}45 \times 10^4$ counts/min per μM	$2{\cdot}04 \times 10^4$	33·6	$1{\cdot}36 \times 10^4$ counts/min per μM
Hypoxanthine	$0{\cdot}27 \times 10^4$	2·5	—	trace	—	—
Xanthine	$1{\cdot}026 \times 10^4$	9·6	—	$2{\cdot}91 \times 10^4$	48	—

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analyzed simultaneously and no discrepancy in the properties of the two substances was found.

The effect of psicofuranine on the synthesis *de novo* of purine bases in *E. coli B* from glycine-1-^{14}C is summarized in Table 2. It may be seen that in the presence of psicofuranine, the synthesis of the purine ring, measured by the incorporation of radioactive carbon from glycine-1-^{14}C, was inhibited by 31 per cent and the distribution of the isotope between the four purine bases was changed. While the percentage incorporation of the isotope into adenine remained practically unchanged, a significant decrease in the radioactivity of guanine, together with an increase in the radioactivity of xanthine was observed. Table 2 also shows that the incorporation of glycine-1-^{14}C into proteins was only slightly inhibited in the presence of psicofuranine under these experimental conditions. Table 3 shows the effect of psicofuranine on the conversion of hypoxanthine-8-^{14}C into other purines in *E. coli B*. The results are analogous to those obtained when glycine-1-^{14}C was used as tracer. The percentage radioactivity incorporated into adenine was the same as the control, the radioactivity of guanine was decreased and the radioactivity of xanthine increased, both to an extent comparable to the previous experiment. Conversion of xanthine-8-^{14}C into other purines was studied similarly and the results are shown in Table 4. In this case, the conversion of xanthine to both adenine and guanine was inhibited by the presence of psicofuranine.

DISCUSSION

The last stages of the biosynthesis of purine bases at the nucleotide level[19, 20] are schematically shown below:

adenylosuccinate → adenosine-5′-phosphate

inosine-5′-phosphate

xanthosine-5′-phosphate → guanosine-5′-phosphate

The reversal of the inhibitory action of psicofuranine on bacterial growth specifically by guanine and its derivatives, the other purines and pyrimidines being ineffective in this respect, could indicate the inhibition of G5′P synthesis from X5′P. Detailed experiments with guanine in concentrations varying from 0 to 10 μg/ml showed (Fig. 3) that with 5 μg/ml of guanine, a complete reversal of the inhibition by psicofuranine was achieved and larger amounts of guanine in the medium had no additional effect on growth. When the nutritional requirements of the purineless mutant *E. coli B*-96 (obtained from Dr. S. Friedman, New York Medical College, New York) were examined, it was found that 7·5 μg of guanine per ml ensured maximal growth. Burton[21] in his work with this mutant supplemented the medium with 7 μg/ml of guanine. Considering that (a) in the mutant, the added guanine must also provide for the adenine requirements of the cells and that (b) the postulated block in the synthesis of G5′P might not be absolute, then the two figures (5 μg/ml with inhibited cells and 7·5 μg/ml with the mutant) are in reasonable agreement. Additional support for the assumption that psicofuranine inhibited the amination of X5′P [22] comes from the observation that the cells growing in the presence of psicofuranine excreted xanthosine into the medium. Accumulation of xanthosine in the medium was reported by Magasanik and Brooke[23] with an *Aerobacter aerogenes* mutant which specifically lacked the X5′P aminase. Apparently, the cells disposed of the accumulating X5′P by excreting it as the nucleoside. It is probable that other nucleotides are also

excreted as nucleosides, since Friedman and Moat[24] found accumulation of 5-aminoimidazole riboside and inosine in the medium by biotin-deficient yeast.

The data obtained with glycine-1-^{14}C and hypoxanthine-8-^{14}C as isotopically labeled precursors of adenine and guanine are entirely in agreement with the assumption that psicofuranine inhibits the amination of X5′P to G5′P. The percentage distribution of the radioactive carbon from both tracers (Tables 2 and 3) into the purine bases showed a decrease in the radioactivity of guanine with a simultaneous increase in the radioactivity of xanthine; the radioactivity of adenine remained the same. However, the total synthesis of the purines was inhibited (31 per cent in the glycine-1-^{14}C experiment and 42 per cent in the hypoxanthine-8-^{14}C experiment) and this was reflected in the specific activities of both adenine and guanine. It may be noted that the decrease in specific activities of guanine was considerably larger than the decrease in the specific activities of adenine. This result lends support to the suggestion made by Magasanik[25] that the relative concentrations of ATP and GTP control the synthesis of A5′P and G5′P, respectively, from the common precursor I5′P. ATP has been shown to participate in the amination of X5′P to G5′P [22] and to inhibit the reductive deamination of G5′P to I5′P. [26] On the other hand, GTP is required for the condensation of I5′P with aspartic acid to adenylosuccinic acid, an immediate precursor of A5′P. [27] Consequently, the inhibition of G5′P synthesis would result in a decrease in GTP level and a subsequent decrease in the synthesis of A5′P. The data, showing decreases in the specific activities of adenine and guanine, are in harmony with this theoretical conclusion.

The data obtained with xanthine-8-^{14}C (Table 4) again showed that in the presence of psicofuranine, the synthesis of G5′P was inhibited with the resulting accumulation of X5′P. The percentage incorporation of the isotope into adenine in the presence and absence of psicofuranine followed that expected from the properties of the isolated enzymes. I5′P dehydrogenase[28] and X5′P aminase.[22] Both enzyme reactions are practically irreversible and therefore the conversion of X5′P to A5′P must be via G5′P, followed by reductive deamination to I5′P,[26] condensation with aspartic acid and hydrolysis to A5′P. Thus the inhibition of X5′P aminase by psicofuranine should not only decrease the conversion of xanthine-8-^{14}C to guanine but also the subsequent conversion to adenine. The relative radioactivities of adenine (37·6 per cent in the absence and 18·5 per cent in the presence of psicofuranine) agreed with this interpretation.

Magee and Eberts[29] studied the effect of psicofuranine on the incorporation of glycine-1-^{14}C into the purines and proteins of the Walker carcinoma of the rat. They observed decreases in specific activities of adenine and guanine comparable to those found in *E. coli B* (Table 2) and a pronounced decrease in the specific activity of the proteins. As Table 2 shows, no marked change in the specific activity of proteins in *E. coli B* was noted in the presence of psicofuranine. This apparent difference between the tumor and the microbial system can be explained by the fact that the bacterial suspension used was more concentrated (transmittance 20 per cent) than the culture at the maximal growth (transmittance approximately 30 per cent). Thus, no growth was possible and presumably only exchange reactions between the labeled glycine and the protein were taking place.

Acknowledgements—The writer is grateful to Drs. K. M. Mann, W. E. Magee, F. S. Eberts and L. J. Hanka for their stimulating discussions. Thanks are due to Dr. Magee for performing the column chromatography experiment.

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THE PHARMACOLOGY AND TUMOUR GROWTH INHIBITORY ACTIVITY OF 1-AMINO*CYCLO*PENTANE-1-CARBOXYLIC ACID AND RELATED COMPOUNDS

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(*Received* 20 *May* 1960)

Abstract—The tumour growth inhibitory activity and toxicity of a number of alicyclic α-amino acids and related compounds has been investigated. Compounds studied include amino acid derivatives of *cyclo*propane, *cyclo*butane, *cyclo*pentane, *cyclo*hexane, *cyclo*heptane, and *cyclo*octane. Significant anti-tumour activity was only exhibited by compounds closely related to 1-amino*cyclo*pentane-1-carboxylic acid. The pharmacological properties of a number of the compounds examined are discussed.

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INTRODUCTION

The tumour growth inhibitory activity of 1-amino*cyclo*pentane-1-carboxylic acid has been briefly reported.[1] Some pharmacological, toxicological, and anti-tumour properties of a series of derivatives and analogues of this active cyclic amino acid are dealt with in the present communication. The preparation and chemical properties of the new compounds examined have been reported elsewhere.[2]

EXPERIMENTAL

Materials

All compounds tested were synthesised in our laboratories. We are indebted to Dr. Howard Bond of the Cancer Chemotherapy National Service Center of the National Institutes of Health, Bethesda, U.S.A., for making available an extra supply of 1-amino*cyclo*pentane-1-carboxylic acid. For the biological tests the compounds, unless otherwise stated, were administered to the rats by the intraperitoneal route and were dissolved in water or suspended in arachis oil.

Animals and diets

Male and female rats from an inbred Wistar colony were used. Their normal food consisted of "rat cake", a commercial product of the following composition:

	(%)
Fine bran	17·4
Ground wheat	17·4
Sussex ground oats	17·4
Ground maize	8·7
Ground barley	8·7
White fish meal	4·8
Meat and bone meal	9·6
Dried skimmed milk	14·0
Dried yeast	1·2
Salt	0·4
Cod liver oil	0·4

In some toxicity experiments animals were fed on a "soft mash" diet consisting of:

	(%)
Water-soaked bread	75·0
Milk powder	6·0
Casein	5·0
Bemax	4·0
Bran	4·0
Dried yeast	3·0
Salt	1·0
Cod liver oil	1·0
Chalk	1·0

5, 10 and 20 per cent protein diets were also used in some tests.*

The animals undergoing the Walker tumour tests were maintained on a varied diet which has been used routinely in our animal colony for many years. Over a 7-day period they have "rat cake" on two non-consecutive days and on other days have bread and milk, bread and margarine, bread and marmite, bread and cod liver oil, and oats.

Toxicity determinations

Preliminary toxicity tests were carried out on groups of three female rats. The animals were about 6–7 weeks old and weighed about 200 g. They were fed on rat cake diets only and the compounds were administered intraperitoneally in water except where otherwise stated (see Tables 1–6). Each animal was weighed daily for at least 4 days before injection of a test compound. Weighing was continued for a further 12–14 days and the growth curve plotted. Weight loss (L_1) was assessed as described by Elson[3] by comparing the actual weight reached with that which would have been attained if the animal had continued its pre-injection steady rate of growth.

* The composition of the 5 per cent protein diet is: casein 5 per cent, starch 85 per cent, Bemax 2·5 per cent, margarine 5 per cent, chalk 0·5 per cent, Salt mixture 1 per cent, Cod liver oil 1 per cent. For the 10 and 20 per cent protein diets the casein is increased by 5 and 15 per cent and starch reduced by the corresponding amounts.

Post-mortem examinations were carried out on animals which died and on survivors killed after the 12–15 day test period. Histological examinations were made on sections of various organs in some cases.

Tumour inhibition experiments

The tests for inhibition of Walker rat carcinoma 256 were carried out on male rats essentially as described by Haddow *et al.*,[4] 6 animals being used in each of the control and test groups. Treatment was started on the day following implantation of the tumour and except where otherwise stated (see Tables 1–6) the compounds were injected intraperitoneally in aqueous solution or occasionally suspended in arachis oil. A few experiments have also been carried out with oral administration of aqueous solutions. In assessment of the degree of inhibition the ratio *C/T* refers to the total weight of the tumours of the control group divided by that of the treated group when the animals are killed and the tumours dissected out and weighed: this is usually done 10 - 12 days after implantation.

Blood counts

Serial blood counts have been carried out on rats treated with 1-amino*cyclo*pentane-1-carboxylic acid (CB 1639). Blood was taken from a tail vein direct into the pipette in which it was diluted in the usual manner. Haemoglobin was estimated photocolorimetrically on a suitably diluted solution.

RESULTS

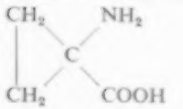
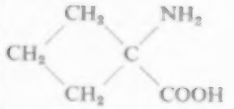
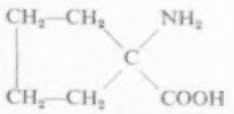
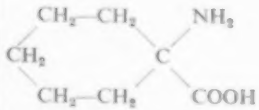
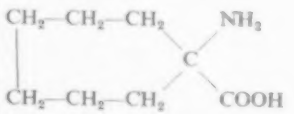
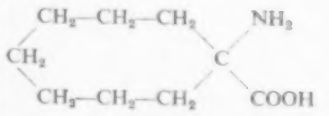
Tumour growth inhibition and toxicity

Table 1 shows toxicity data and activity against the Walker rat carcinoma 256 of a series of alicyclic α-amino acids, in which the ring size has been varied from 3 to 8 carbon atoms. Tumour growth inhibitory activity has only been shown by the *cyclo*pentane derivative (CB 1639) which is also the most toxic compound of the series.

Although a single dose of 250 mg of CB 1639 per kg caused prolonged growth inhibition in normal rats this dose had no appreciable effect on tumour growth. Six consecutive daily doses of 125 mg/kg started on the day following implantation and given either intraperitoneally or by forced feeding caused definite inhibition of the growth of the tumour: this effect was still apparent, though less marked, with a dose of 75 mg/kg given similarly during 8 days. In one experiment the transplanted Walker carcinoma was allowed to grow for 7 days before treatment began; no inhibition of growth occurred when a dose of 75 mg/kg was given on the 6 following days. CB 1639 also checked the growth of the more resistant Crocker sarcoma 180 in the mouse. A *C/T* ratio of 5·7 was obtained in groups of 5 animals—the treated animals receiving five daily injections of 2 mg per 25 g mouse of amino acid in 0·2 ml of water. The compound was toxic to mice at double this dose. When C^- Bagg Albino mice bearing a first transplant from a C^+ mouse mammary tumour were treated with eleven doses of 2 mg of CB 1639 per mouse given on alternate days a *C/T* ratio of 2·85 was obtained.

Table 2 shows the results obtained with a few ring substituted derivatives of the *cyclo*pentane and *cyclo*hexane amino acids. The introduction of a carboxyl group into CB 1639 leads to the lowering of toxicity but also to the loss of anti-tumour

TABLE 1

Code no.	Compound	Structure*	Activity against Walker tumour				Toxicity data	
			Dose (mg/kg)	Time (days)	Result	C/T	Single dose (mg/kg)	Deaths within 2 weeks
CB. 1703	1-Aminocyclopropanecarboxylic acid		250	5 (alternate)	−ve		500	0/3
1700	1-Aminocyclobutanecarboxylic acid		500	5 (alternate)	−ve		500	0/3
1639	1-Aminocyclopentanecarboxylic acid		500	3	toxic		♀1000	3/3
			250	1	low	1·29	♀ 750	3/3
			125	6	+ve	30·5	♀ 500	3/3
			125 f.f.*	6	+ve	10·3	♀ 375	7/8
			75	8	+ve	3·0	♀ 250	1/6
			75 f.f.	8	+ve	3·6	♀ 200	0/3
							♂ 250	7/9
							♂ 150	0/6
1641	1-Aminocyclohexanecarboxylic acid		500	12	−ve		1000	0/3
							500	0/3
1692	1-Aminocycloheptanecarboxylic acid		500	4 (alternate)	−ve		500	0/3
1714	1-Aminocyclooctanecarboxylic acid		500	4 (alternate)	−ve		500	0/3

* Forced feeding

Table 2

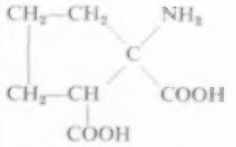
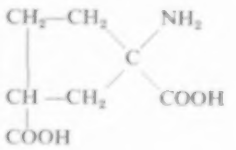
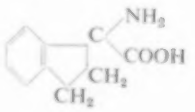
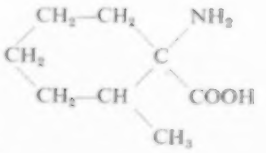
Code no.	Compound	Structure	Activity against Walker tumour				Toxicity data	
			Dose (mg/kg)	Time (days)	Result	*C/T*	Single dose (mg/kg)	Deaths within 2 weeks
1701	1-Amino*cyclo*pentane-1 : 2-dicarboxylic acid		500	5 (alternate)	−ve		500	0/3
1712	1-Amino*cyclo*pentane-1 : 3-dicarboxylic acid		500	4 (alternate)	−ve		500	0/3
1696	1-Amino-2 : 3-benz*cyclo*pentane-1-carboxylic acid		250	8	low	1·3		
1645	1-Amino-2-methyl*cyclo*hexane-carboxylic acid		500	10	−ve		500	0/3

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TABLE 2—*continued.*

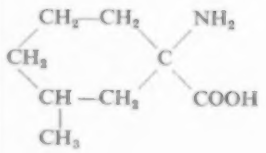
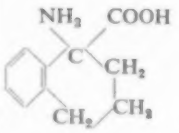
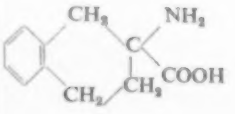
Code no.	Compound	Structure	Activity against Walker tumour				Toxicity data	
			Dose (mg/kg)	Time (days)	Result	*C/T*	Single dose (mg/kg)	Deaths within 2 weeks
1638	1-Amino-3-methyl*cyclo*hexane-carboxylic acid		500	8	−ve		1000	0/3
1643	1-Amino-2 : 3-benz*cyclo*hexane-1-carboxylic acid		500	12	−ve		500	0/3
1647	1-Amino-3 : 4-benz*cyclo*hexane-1-carboxylic acid		500	10	−ve		500	0/3

Table 3

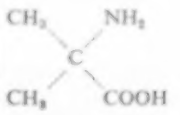
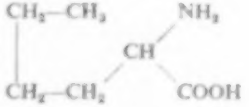
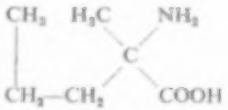
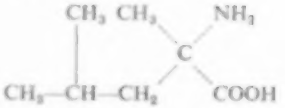
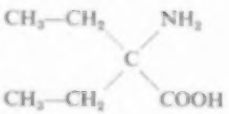
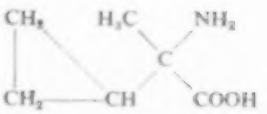
Code no.	Compound	Structure	Activity against Walker tumour				Toxicity data	
			Dose (mg/kg)	Time (days)	Result	*C/T*	Single dose (mg/kg)	Deaths within 2 weeks
CB 1637	α-Methylalanine		1000	9	−ve		2500	0/3
1688	1-Aminopentanecarboxylic acid (DL-norleucine)		250	9	−ve			
1685	2-Aminopentane-2-carboxylic acid		500	8	−ve		500	0/3
1640	α-Methyl-leucine		1000	10	−ve		1000	0/3
1686	3-Aminopentane-3-carboxylic acid		500	9	low	1·5	500	0/3
1653	α-*cyclo*Propylalanine		250	7	low	1·36	500 250 500 (oil) 250 (oil)	2/3 0/3 3/3 0/3

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TABLE 4

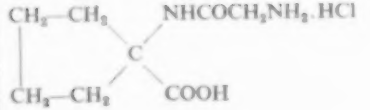
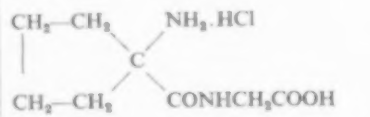
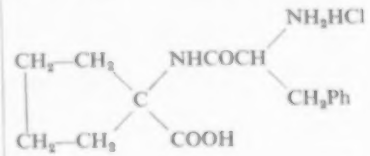
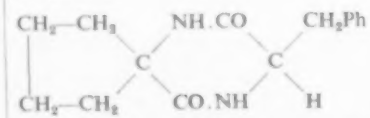
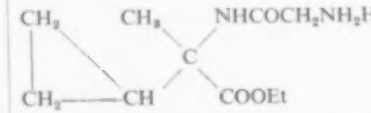
Code no.	Compound	Structure	Activity against Walker tumour				Toxicity data	
			Dose (mg/kg)	Time (days)	Result	*C/T*	Single dose (mg/kg)	Deaths within 2 weeks
CB 1659	Hydrochloride of glycyl-1-amino-*cyclo*pentane-1-carboxylic acid		250 125	6 8	+ve +ve	9 5·3	500	0/3 L_1 70 g
1668	Hydrochloride of 1-amino-1-(N-carboxymethyl)carboxyamido-*cyclo*pentane		125	10	+ve	7·8	500	0/3
1677	Hydrochloride of DL-phenylalanyl-1-amino*cyclo*pentanecarboxylic acid		100	8	−ve		250	0/3
1690	3-Benzyl-6 : 6-tetramethylene-2 : 5-dioxopiperazine		250	5 (alternate)	−ve		500	0/3
1706	Hydrochloride of glycyl-α-*cyclo*-propylalanine-ethyl ester		500	8	−ve		1000 500	0/3 0/3

TABLE 4—*continued.*

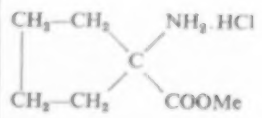
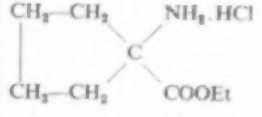
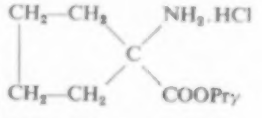
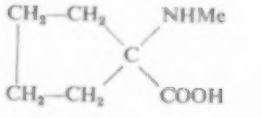
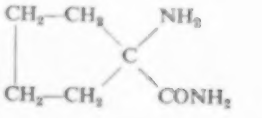
Code no.	Compound	Structure	Activity against Walker tumour				Toxicity data	
			Dose (mg/kg)	Time (days)	Result	*C/T*	Single (mg/kg) dose	Deaths within 2 weeks
1704	Hydrochloride of methyl-1-amino-*cyclo*pentanecarboxylate		250	5	Toxic		500	3/3
1691	Hydrochloride of ethyl-1-amino-*cyclo*pentanecarboxylate		375 (oil) 250	1 5	+ve Toxic	4·7	750 (oil) 500	0/3 L_1 44 g 1/3 L_1 100 g
1708	Hydrochloride of *iso*propyl-1-amino-*cyclo*pentanecarboxylate		250 125 50	5 6 10	Toxic Toxic +ve	2·3	500	0/3
1728	1-Methylamino*cyclo*pentane-carboxylic acid		250	1	−ve		375	0/3
1705	1-Amino-1-carbamido*cyclo*pentane		125	6	+ve	7·4	500 375 250	3/3 3/3 0/3

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TABLE 4—*continued.*

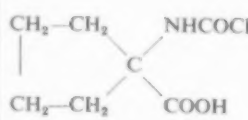
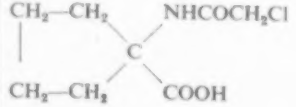
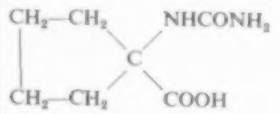
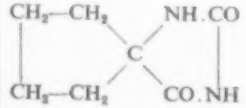
Code no.	Compound	Structure	Activity against Walker tumour				Toxicity data	
			Dose (mg/kg)	Time (days)	Result	*C/T*	Single dose (mg/kg)	Deaths within 2 weeks
1709	1-Acetylaminocyclopentane-carboxylic acid		250	9	low	1·3	500 250	0/3 0/3
1732	1-Chloracetylaminocyclopentane-carboxylic acid		500	1	−ve		500	0/3
1702	1-Ureidocyclopentanecarboxylic acid		500	9	−ve		500 (in bicarb.)	0/3
1683	5 : 5-Tetramethylenespirohydantoin		500 (oil)	8	−ve		1000 (oil)	0/3

TABLE 5

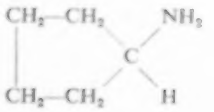
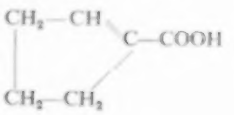
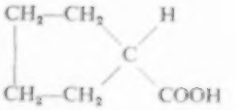
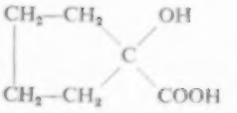
Code no.	Compound	Structure	Activity against Walker tumour				Toxicity data	
			Dose (mg/kg)	Time (days)	Result	*C/T*	Single dose (mg/kg)	Deaths within 2 weeks
1689	*cyclo*Pentylamine		25 (oil)	9	−ve		500 250 100 50	3/3 3/3 3/3 0/3 L_1 27 g
1684	*cyclo*Pent-1-enecarboxylic acid		500 (bicarb.)	8	−ve		500 (bicarb.) 250 (bicarb.) 125 (bicarb.) 250 (water)	0/3 0/3 0/3 2/3
1725	*cyclo*Pentanecarboxylic acid		500 (bicarb.)	8	−ve			
1693	1-Hydroxy*cyclo*pentanecarboxylic acid		250 125	2 7	toxic low	1·4	500	1/3

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TABLE 5—*continued.*

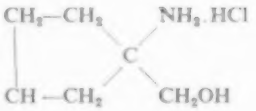
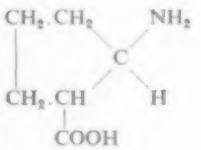
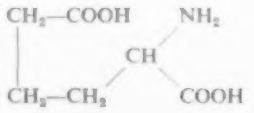
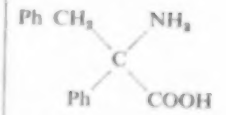
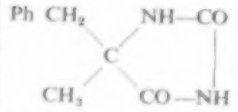
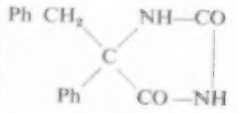
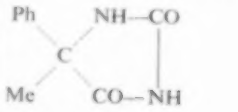
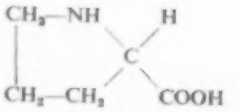
Code no.	Compound	Structure	Activity against Walker tumour				Toxicity data	
			Dose (mg/kg)	Time (days)	Result	*C/T*	Single dose (mg/kg)	Deaths within 2 weeks
1722	1-Hydroxymethylpentylamine hydrochloride		500	4	−ve			
1713	2-Aminocyclopentanecarboxylic acid		250	9	−ve		500	1/5
1710	α-Aminoadipic acid		500 (bicarb.)	6	−ve		500	0/3

TABLE 6

Code no.	Compound	Structure	Activity against Walker tumour				Toxicity data	
			Dose (mg/kg)	Time (days)	Result	C/T	Single dose (mg/kg)	Deaths within 2 weeks
CB 1636	Hydantoic acid		125 f.f.	8	−ve		1000 500 250	3/3 2/3 0/3
1654	DL : β-Diphenylalanine		500 (oil)	3	−ve		500 (oil)	0/3
1665	5-Benzyl-5-methylhydantoin		250 (oil)	7	−ve		500 (oil)	0/3
1715	5-Benzyl-5-phenylhydantoin		250	4 (alternate)	−ve		250	0/3
1664	5-Methyl-5-phenylhydantoin		125 (oil)	7	−ve		500 (oil) 250 (oil)	3/3 0/3
1707	L-Proline		500	6	−ve			

activity. The compound obtained by fusing a benzene ring on to the molecule of CB 1639—1-amino-2 : 3-benz*cyclo*pentane-1-carboxylic acid (CB 1696)—shows inhibitory activity of a very low order. No activity was shown by the methyl or benz-substituted cyclohexane amino acids.

Having established the unique character of the five-membered ring system it was then of interest to decide whether the cyclic structure was of importance—it will be realized that this work began as an investigation into α-substituted non-cyclic amino acids.[1] Accordingly compounds were examined (Table 3) which represented ring opening between C_1 and C_2 (CB 1688), C_2 and C_3 (CB 1685), and between C_3 and C_4 (CB 1686). Only the last compound showed any inhibitory activity and this was of a low order compared with the cyclic compound. A compound (CB 1653) in which ring closure of the five carbon chain was effected between C_2 and C_4 also showed slight anti-tumour activity. A single dose of 250 mg/kg of CB 1653 caused significant weight loss in the normal rat.

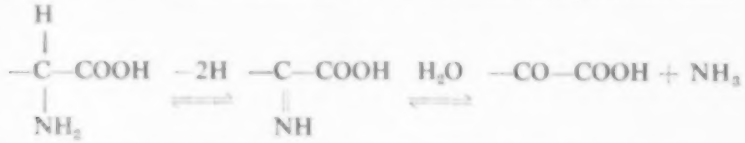
As it had now been established that appreciable activity was only associated with the *cyclo*pentane structure numerous derivatives of CB 1639 were next examined (Table 4). Since there is evidence[5] that the anti-metabolic activity of an amino acid analogue can be enhanced by incorporation into a peptide structure the preparation for test of two glycine and two phenylalanine peptides of 1-amino*cyclo*pentane-1-carboxylic acid was attempted.[2] Both glycine peptides (CB 1659 and CB 1668) showed activity comparable with that of the parent amino acid and were considerably less toxic. On the other hand, the phenylalanine peptide (CB 1677) and the corresponding cyclic dipeptide (CB 1690) showed no activity. A glycine peptide of *cyclo*propylalanine (CB 1706) was inactive against the transplanted tumour though this peptide retained the ability possessed by the parent acid (CB 1653) to retard the growth of the normal rat.

A number of derivatives of 1-amino*cyclo*pentane-1-carboxylic acid which could readily yield the amino acid *in vivo* was studied: it has been established that derivatives of cytotoxic agents with so-called latent activity[6] are often of interest since they constitute a transport form with physical properties differing from those of the parent agent.Three esters which would hydrolyse at different rates to give the amino acid were examined. The most readily hydrolysed methyl ester (CB 1704) proved to be the most toxic and the more stable *iso*propyl ester (CB 1708) the least toxic. In general there appeared to be no particular advantage in using the esters of CB 1639 as far as tumour growth inhibition was concerned.

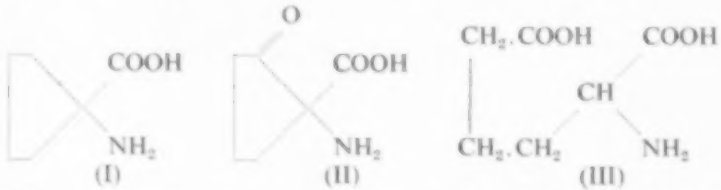
Two amides, in which the carboxyl group (CB 1705) or the amino group (CB 1709) were masked, were tested. The compound containing the free amino group was more toxic and exerted the greater effect on the tumour. Derivatives possessing a free amino group often show greater activity as indicated by the retention of activity in the glycine peptides and in similar peptides of melphalan.[7] It has been shown[8] that some hydantoins are hydrolysed *in vivo* to give the corresponding amino acids and accordingly the spirohydantoin (CB 1683) and the intermediate hydrolysis product (the ureido acid, CB 1702) were examined. These compounds proved to be ineffective and it can be assumed that no significant amounts of the amino acid were, in fact, formed.

The importance of the five-membered ring structure for anti-tumour activity prompted a consideration of differences between 1-amino*cyclo*pentane-1-carboxylic

acid and closely related but inactive homologues. One metabolic pathway followed by α-amino acids involves oxidation and reversible amination processes thus:



This pathway is not available for α-substituted α-amino acids since the formation of the imino acid is precluded. However β-oxidation, such as occurs with linear fatty acids, can proceed and by this route CB 1639 (I) would give the cyclic keto acid (II):



Ring fission of (II) should proceed readily to give α-aminoadipic acid (III), a compound which accumulates during abnormal lysine metabolism.[9] There is reason to believe that keto acids of type (II) derived from *cyclo*hexane would less readily undergo ring fission[10] whilst the products derived from the three- and four-membered rings would be the common amino acids, aspartic acid and glutamic acid, respectively. This hint of specific property of 1-amino*cyclo*pentane-1-carboxylic acid led to the synthesis and testing of α-aminoadipic acid which, however, proved to be ineffective.

Other obvious transformation products of CB 1639 are *cyclo*pentylamine, formed by the loss of carbon dioxide, and *cyclo*pent-1-ene-1-carboxylic acid, formed by the loss of ammonia. The amine proved to be surprisingly toxic (Table 5) but at a tolerated dose it had no effect on tumour growth. *cyclo*Pent-1-ene-1-carboxylic acid was also quite toxic when administered in water but it was well tolerated when given in aqueous sodium bicarbonate. The acid had no effect on tumour growth. It was felt that the stomach ulceration caused by CB 1639 might be associated with the local formation of this unsaturated acid since such irritation is not uncommon with unsaturated compounds.[11] Two other compounds which should be readily converted into the unsaturated acid are 1-hydroxy*cyclo*pentane-2-carboxylic acid (an isostere of CB 1639) and 1-amino*cyclo*pentane-2-carboxylic acid, this β-amino-acid loses ammonia even more readily than the tertiary α-amino-acid. The hydroxy-acid (CB 1693) was quite toxic and showed tumour growth inhibitory activity of a low order. 1-Amino*cyclo*-pentane-2-carboxylic acid (CB 1713) showed no appreciable anti-tumour activity but liver congestion and adhesions were observed in treated animals. Recently it has been shown that the amino-acid (CB 1713) which was submitted for test was the *trans*-isomer.[12]

Table 6 lists some miscellaneous compounds examined in the course of this work. None of these substances caused inhibition of the growth of the transplanted tumour, but there was evidence that several of the hydantoins caused retardation of growth in normal rats.

As the work progressed it became apparent that only compounds closely related to 1-amino*cyclo*pentane-1-carboxylic acid were effective as tumour growth inhibitors

and in order to obtain information about the mode of action a more detailed study of the effects produced by this compound was undertaken.

Pharmacological investigations of 1-*amino*cyclo*pentane*-1-*carboxylic acid* (*CB* 1639)

(a) *Haematological effects.* In preliminary haematological tests a single dose of 750 mg/kg of CB 1639 dissolved in water was administered by intraperitoneal injection to 4 male rats. This dose proved to be toxic as 3 rats died within 9 days of the injection. The weight and blood response curves of the survivor together with those of one of the animals which died on the ninth day after treatment are shown in Fig. 1

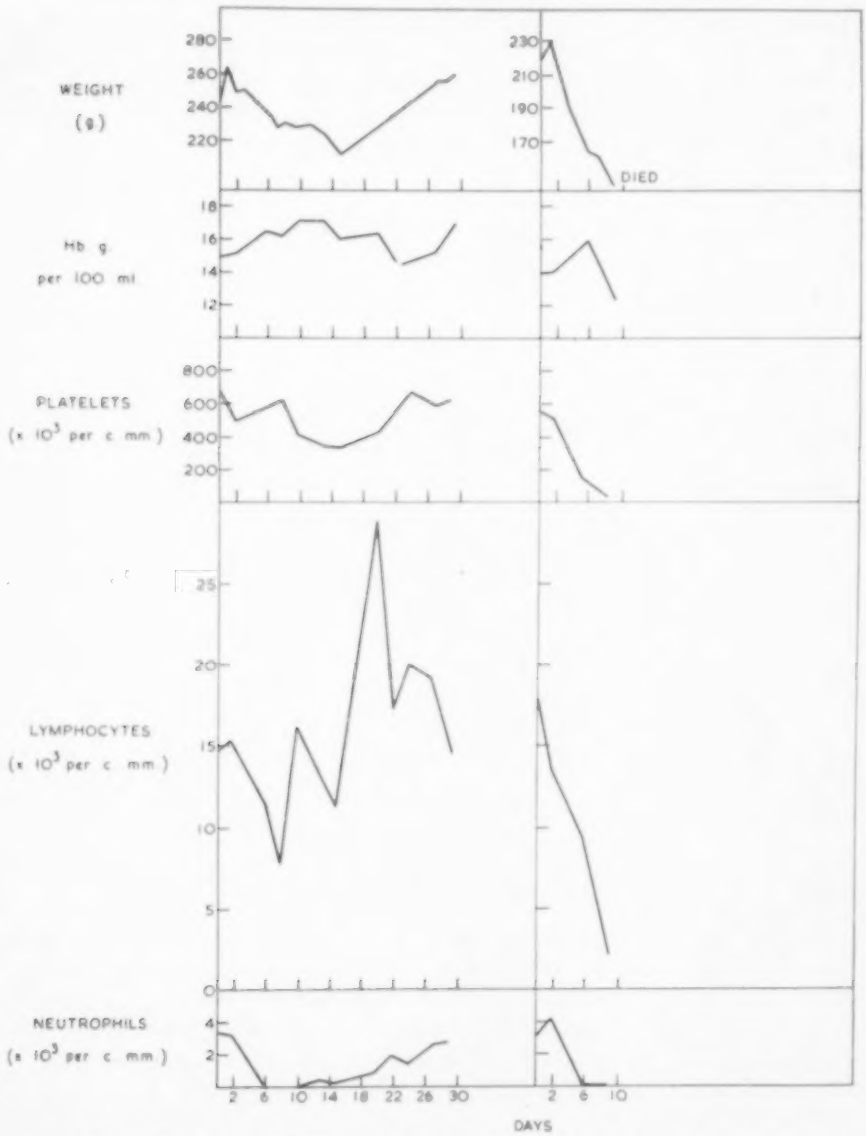


FIG. 1. Effect of CB 1639 (750 mg/kg i.p.) on the weight and blood of male rats.

The greatest effect is seen to be on the neutrophils which in both cases had completely disappeared from the circulation by the sixth day. In the case of the survivor small numbers of neutrophils began to reappear in the peripheral blood on the twelfth day and the numbers gradually increased until normal values were again attained about 30 days after the original injection. In the 3 animals that died no reappearance of neutrophils was observed, and the other blood elements all fell precipitously until death ensued.

At post-mortem of the rat dying at 9 days, only slight pathological changes related to the thrombocytopenia were observed. Superficial lymph nodes showed only minimal evidence of haemolymph change and there were a few small petechial haemorrhages in the skin. The adrenal glands were very dark and haemorrhagic. The main cause of death, however, was probably haemorrhage in the stomach which was filled with fresh blood and showed inflammation and haemorrhage in the pyloric portion. The thymus was small; heart and lungs appeared normal.

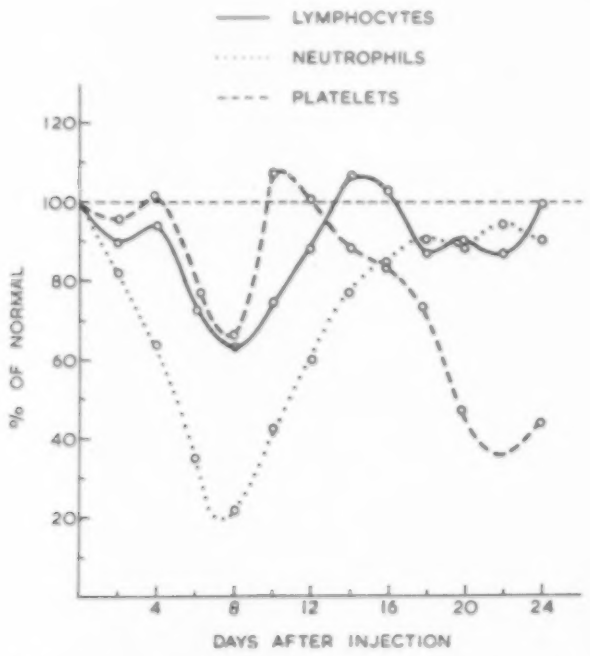


Fig. 2. Blood response pattern in male rats to CB 1639 (200 mg/kg i.p.).

Following this experiment, blood counts were carried out on 4 male rats treated with a non-lethal dose (200 mg/kg intraperitoneally). This dose caused some weight loss for a few days after the injection ($L_1 = 35$ g) but no other obvious detrimental effects. Figure 2 shows the percentage change from normal for lymphocytes, neutrophils, and platelets in the circulating blood. The curve represents the mean values from 4 rats. Again the main effect is on neutrophils which fall to 20 per cent of their normal value in about 7 days after the single injection of CB 1639. Recovery is then steady and normal values are regained after a further 8 to 10 days.

(b) *Growth inhibition and toxicity*. The most marked physiological effect of 1-aminocyclopentane-1-carboxylic acid (CB 1639) is a prolonged inhibition of growth. This appears to be largely, though not entirely, due to loss of appetite and reduced food and water intake. Figure 3 shows the effect of CB 1639 (375 mg/kg administered intraperitoneally) on the weight of 6 female rats maintained on a "hard" rat cake diet and on nine fed a "soft mash" diet. On the rat cake diet all animals began to lose weight rapidly following the injection. One animal started to regain weight after 6 days and at post-mortem examination after 21 days showed no sign of stomach ulcers. The other 5 rats continued to lose weight steadily until death occurred 16–17 days after the injection. The rats which died all showed areas of haemorrhage and ulceration in the stomach. On the other hand, of the 9 animals fed the soft mash diet only one

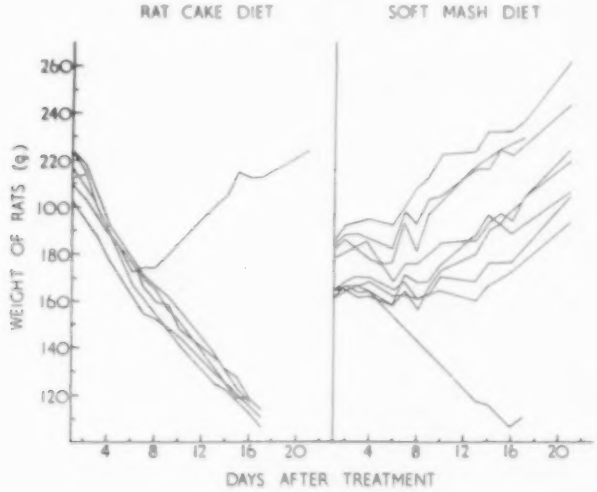


FIG. 3. Effect of CB 1639 (375 mg/kg i.p.) on the weight of female rats maintained on "rat cake" and "soft mash" diets.

showed a prolonged weight loss and stomach lesions. The other 8 rats showed relatively little weight loss and when examined at post-mortem after about 20 days they were free from stomach ulcers.

Some effects of the nature of the food offered on the quantities consumed are illustrated in Fig. 4. This shows the weight of food eaten by 2 male rats injected with CB 1639. A moderate amount of semi-soft food (bread) was eaten, but when this was replaced by "hard" dog biscuit, which is eaten readily by normal rats, only a small amount was consumed on the first day and it was refused on the next day. The soft mash diet given on the following 3 days was eaten in quantity but the dog biscuit was again refused when offered during the next 2 days. Subsequently both "mash" and bread were accepted. It is known that controls within the central nervous system, particularly within the hypothalamus are essential for the normal regulation of spontaneous food and water intake and some of the effects of 1-aminocyclopentane-carboxylic acid might be due to a direct action on the hypothalamus. However, some

experiments carried out by Dr. D. G. Montemurro failed to demonstrate histologically that the effects of CB 1639 were caused by any primary cytotoxic action of the drug on the neurones of the lateral hypothalamic and medial subthalmic areas of the pituitary gland.

Although at first sight it may appear from these observations that the hard or soft nature of the food may be of primary importance in determining the toxicity of CB 1639 this simplified view is not supported by an experiment in which the rats were

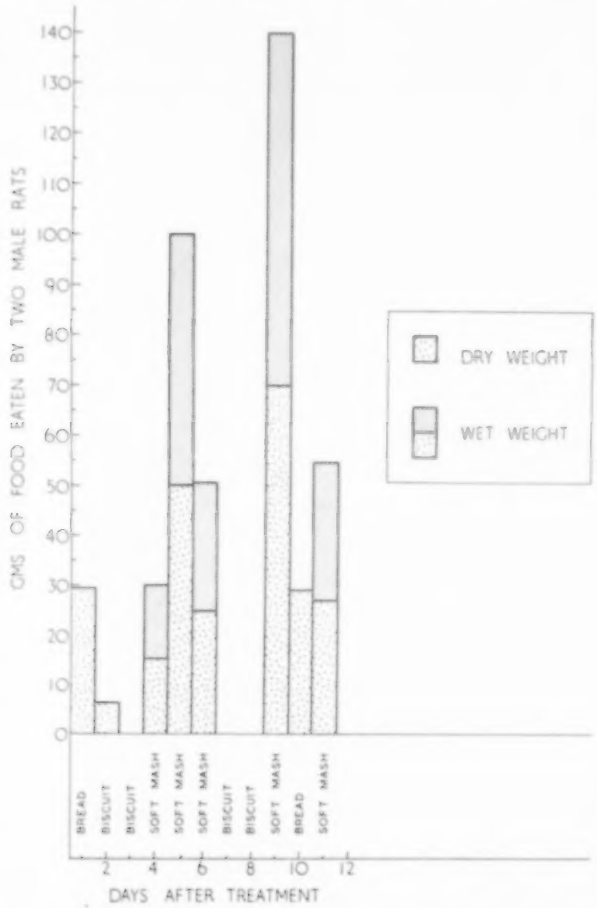


Fig. 4. Effects of the nature of the food offered on the quantities consumed by rats treated with CB 1639 (375 mg/kg i.p).

fed the rat cake in a powdered form made into a "mash" with water. The weight loss and survivals of the animals on this diet did not differ from that of those fed rat cake in its normal "hard" form.

Figure 5 shows the survival times of female rats treated with CB 1639 (375 mg/kg) maintained on "semi-soft" diets containing 5, 10 and 20 per cent protein in the form of casein. The lowest survival time and earliest deaths occurred in the animals on the lowest (5 per cent) protein diet whilst the best survival was obtained on the highest

(20 per cent) protein diet, although this rate is not nearly as good as can be obtained in animals receiving the same dose of CB 1639 and maintained on the "soft mash" diet (Fig. 3)

To investigate the question of what constituent of the "soft mash" diet might be mainly responsible for its beneficial effect comparative experiments were carried out

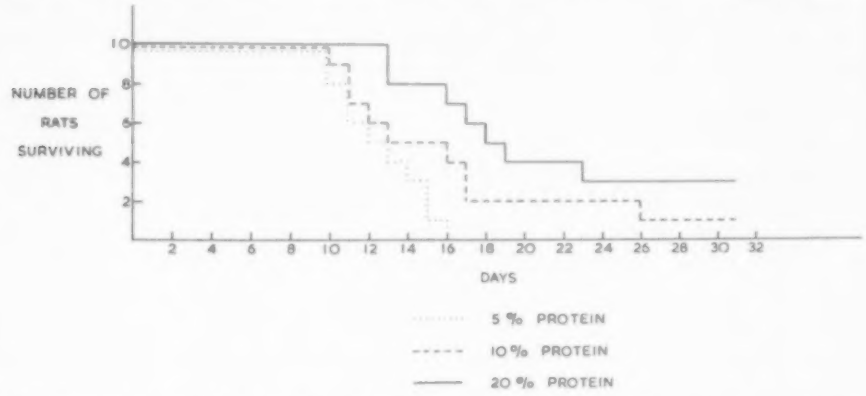


FIG. 5. Survival of female rats treated with CB 1639 (375 mg/kg i.p.) maintained on 5, 10, and 20 per cent protein diets.

with diets of "soft mash", "soft mash" without milk powder, water-soaked bread alone, and water-soaked bread with the addition of milk powder. The results are given in Fig. 6. These were carried out on male rats using a dose of 375 mg/kg. The survival on the soft mash diet is not as good as with female rats treated with the same dose of CB 1639 (Fig. 3) but 5 out of the 10 animals survived on this diet whils

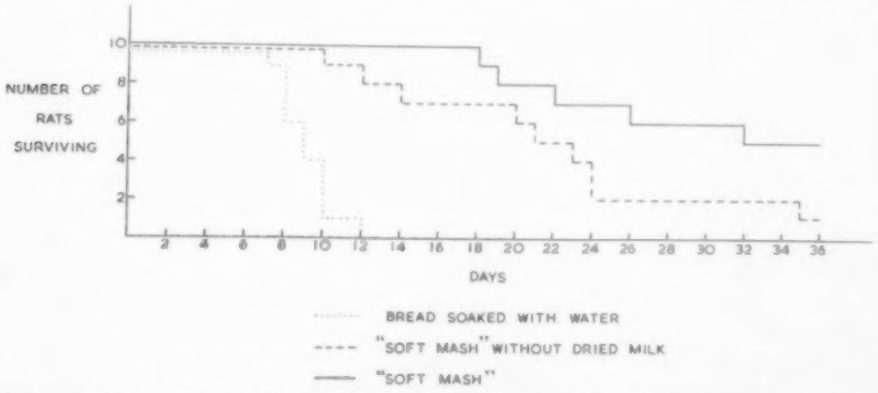


FIG. 6. Survival of male rats treated with CB 1639 (375 mg/kg i.p.) maintained on diets of bread soaked with water, "soft mash" without dried milk, and "soft mash".

there was only one survivor in the group receiving the mash without the addition of milk powder. The bread soaked with water proved to be a very poor diet as all the rats died within 7 to 12 days of the injection of CB 1639.

All rats dying in these groups showed signs of stomach lesions ranging in severity from small haemorrhages to large ulcers. The protective effect of the diet may be

mainly concerned in modifying the severity of these lesions. Obviously a high protein content is of considerable value, but the mere protein content is not the determining factor. It would seem that a "gastric ulcer diet" of soft consistency and a high milk content offers the best protection.

The course of development of stomach lesions in rats receiving CB 1639 is being investigated by pathological and histological examination of the stomachs of rats at frequent intervals after a single intraperitoneal dose of the compound. These results will be published elsewhere.

DISCUSSION

Of all the compounds tested for tumour inhibitory activity only CB 1639 and its ester and peptide derivative showed marked activity. Inhibition of the growth of the Walker carcinoma 256 and some inhibition of the growth of the transplantable mouse sarcoma 180 and of a transplantable mammary tumour in C^{-} Bagg albino mice was demonstrated.

These results are similar to those obtained by Ross *et al.*[13] using the mouse tumours, sarcoma 180, carcinoma 755, and the leukaemia L 1210. Only CB 1639 and its simple derivatives were effective inhibitors and of these tumours the carcinoma 755 showed the greatest response. Marked tumour inhibition is, however, only obtained with doses which cause a considerable body weight loss to the animal.

The tumour growth inhibition, however, cannot be attributed entirely to the general toxic action or body growth inhibitory action of the compound. For instance, *cyclo*-pentylamine (CB 1689) is more toxic than CB 1639 but exhibits no inhibitory action on the Walker tumour.

The influence of nutritional status on the growth of Walker rat carcinoma 256 has been investigated by Walpole[14] who concludes that while compounds which interfere with body growth may be expected to inhibit tumour growth as a result of their general "toxic" action, this inhibition is unlikely to exceed that which would result from underfeeding at a comparable level. Underfeeding at the level required to prevent any increase in mean gross weight of the rat inhibits tumour growth by about 40 per cent. Reduction of food intake to only 3 g per rat per day, which resulted in about 16 per cent loss of body weight over 14 days led to about 60 per cent inhibition of tumour growth—in our method of assessing tumour growth inhibition this would correspond to a C/T ratio of about 2·5. Elson and Haddow[15] and Elson[16] found that maintaining rats on a 5 per cent protein diet made relatively little difference to the weights of Walker tumour compared with controls maintained on a 20 per cent protein diet.

It seems very unlikely, therefore, that C/T ratios as high as 30 obtained with CB 1639 (Table 1) could be attributable solely to a general toxic action causing inhibition of body growth and food consumption.

Like many other tumour-growth inhibitory agents CB 1639 also has a considerable effect on haemopoiesis. The main effect in the case of CB 1639 is a fall in the number of circulating neutrophilis and in this respect it resembles the effect of Myleran and dimethylmyleran[17] or perhaps more closely that of thioguanine.[18] The fall in neutrophils seems to be accompanied by a less drastic fall in the blood platelets than occurs with other compounds so that, but for its toxic properties, causing weight loss and

liability to stomach ulcers, it might have been expected to have shown some promise in the treatment of chronic myeloid leukaemia.

Some clinical trials of CB 1639 in cases of a variety of advanced malignant disease have been very briefly reported.[13, 19] Virtually no beneficial effects were observed.

The mode of action of CB 1639 and the specificity of the five-membered ring structure remain unelucidated. Microbiological experiments have not indicated that the compound is an antagonist of any of the usual amino acids.[20] The formation *in situ* of α-aminoadipic acid by the process described above stands as an attractive possibility. Despite the inactivity of the injected aminodicarboxylic acid, this might still be the effective compound since such an acid would not readily penetrate tumour cells—normal transport mechanisms not operating.[21] If formed within the cell, aminoadipic acid would persist and possibly interfere with lysine metabolism. It would seem well worth while examining some other aminoadipic acid derivatives, such as a range of diesters, which could diffuse into the cells and there release the amino acid.

Acknowledgements—This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Jane Coffin Childs Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service. The writers thank Miss B. Hall for assistance in the animal experiments.

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THE INACTIVATION OF FOLIC ACID ANTAGONISTS BY NORMAL AND LEUKAEMIC CELLS

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(*Received* 27 *May* 1960)

Abstract—(1) The folic acid antagonist aminopterin arrests cell division at the metaphase stage by interfering with the function of folinic acid. Certain cell types overcome this inhibition by inactivating the inhibitor.

(2) The capacity to convert aminopterin into an inactive form is found in chick embryo osteoblasts and fibroblasts, and in red blood cells and buffy coat cells of the adult fowl. Mouse liver can also inactivate aminopterin, but normal bone marrow and normal lymphoblasts and lymphocytes are unable to do so. Leukaemic cells of an acute lymphoblastic mouse leukaemia inactivate aminopterin.

(3) Among human cell types mature lymphocytes do not affect aminopterin but the red blood cells of twenty-one out of twenty-five normal subjects converted aminopterin into an inactive form.

(4) The leukaemic bone marrow of seventeen out of eighteen children with acute leukaemia was found to be a very effective inactivator of aminopterin. This quality resided in no case, whether normal or leukaemic, in the blood plasma or the supernatant fluid.

INTRODUCTION

SINCE folic acid antagonists are useful tools in the analysis of certain biochemical processes[1, 2] and since they can induce complete, though temporary remissions in children with acute leukaemia,[3] it was of interest to investigate further the fate of these compounds when in contact with normal and leukaemic tissue.

Experiments have been reported[4–6] showing that a variety of tissues could inactivate the folic acid antagonist aminopterin. Thus cultures of embryonic chick fibroblasts and osteoblasts and leukaemic cells from mice with acute lymphoblastic leukaemia were able to alter this compound in such a way that it no longer arrested cell division at metaphase. In contrast, normal mouse bone marrow was unable to convert aminopterin into an inactive substance, and chick embryonic intestinal mucous membrane was a rather weak inactivator. Although short-term exposure to aminopterin caused an arrest in metaphase in all tissues tested, different results were obtained when exposure was prolonged; for example, after 24 hr of treatment embryonic chick osteoblasts and fibroblasts in culture went through mitosis with little inhibition, whereas the intestinal mucous membrane *in vivo* remained severely affected for several days. Similarly the effect of aminopterin persisted for days in normal bone marrow cells of man and mouse, while the leukaemic cells almost invariably overcame the initial inhibition.

In the present paper, observations are recorded which add to the range of tissues capable of inactivating folic acid antagonists. In a separate paper[7] experiments will be described which indicate that the inactivation of aminopterin may involve an alteration of the glutamic acid moiety at the end of the side chain of this compound.

MATERIAL AND METHODS

To find whether a tissue was capable of inactivating aminopterin, the following procedure was adopted. Either finely minced tissue fragments or cell suspensions were incubated for 20–24 hr at 37 °C with the folic acid antagonist under strictly sterile conditions. At the end of the incubation period the incubated mixture was centrifuged, and the supernatant was tested for the presence of aminopterin by placing from 0·01 to 0·02 ml on a 48-hr hanging-drop culture of osteoblasts from frontal bones of 12-day chick embryos.

These tissue cultures, which served as the test system, were grown in a thinly spread clot of about 0·02 ml, made of equal parts of cock plasma and chick embryo extract; the explants contained many cell divisions. Great care was taken over two points: (1) that the small quantity of test fluid was placed directly over the area of the cells, and (2) that each culture was outside the incubator for not more than 30 sec while this manipulation was completed. Longer periods at room temperature may affect the dividing cells.

Fifteen minutes after the application of the aminopterin solutions, the cultures were fixed in methanol and stained with May–Grunwald and Giemsa's dyes, according to Jacobson (1954*a*, p. 605–606) and were mounted in a synthetic medium, like DPX (Canada balsam is not suitable).

Mitoses were counted by systematically scanning the zone of outgrowth and the explant of the hanging-drop cultures and were classified into four stages defined as follows. Prophase (P), the chromosomes were still confined within the nuclear membrane; metaphase (M), the chromosomes were in the process of arranging themselves or were already arranged in a single group; anaphase (A), the chromosome halves (the chromatids) were parting or had separated; telophase (T), the chromosome group for each daughter cell had acquired a nuclear membrane. The distribution pattern of these four mitotic phases was recorded as a percentage of all mitoses seen. In a normal culture 35–45 per cent of the dividing cells are in metaphase and 20–25 per cent in anaphase.

In each experiment the following two controls were generally made. (a) The aminopterin control: a solution of aminopterin (usually 0·5 mg/ml) incubated without tissue, in either Tyrode's solution alone or a mixture of Tyrode and serum. These aminopterin controls retained their full inhibitory power and after 15 min application to the test cultures about 60–70 per cent of all dividing cells were in metaphase with severely clumped chromosomes and 0–10 per cent in anaphase. (b) The tissue control: the supernatant of cells incubated without aminopterin was applied for 15 min. It never inhibited mitoses, and tissue cultures to which it had been added showed the normal distribution pattern of metaphases (about 40–45 per cent) and anaphases (20–25 per cent). The tissue whose inactivating power was to be investigated was incubated in a similar solution containing aminopterin, and the supernatant was tested on sister cultures to those used for the control tests (a) and (b). If the tissue had

inactivated the aminopterin, its supernatant produced no change in the normal distribution pattern of mitotic phases. Thus each experiment had its own internal standards of active aminopterin and normal controls. The details of the various experimental procedures employed in the different parts of the investigation will be found at the beginning of each section.

EXPERIMENTS

I. *The So-called "Resistance" of Certain Cell Types to Folic-acid Antagonists*

Object of experiments

As already reported (Jacobson, 1954b), after prolonged treatment with aminopterin some tissues recovered from their mitotic inhibition but others did not. There seemed to be two alternative explanations of this "resistance" to aminopterin: (a) The "resistant" type of tissue might by-pass the block established by aminopterin. Since it has been found that aminopterin replaces folinic acid, this would mean that division could proceed without the functioning of folinic acid or its derivatives. (b) The antagonist might be broken down or inactivated. Experiments were made to find which alternative was correct.

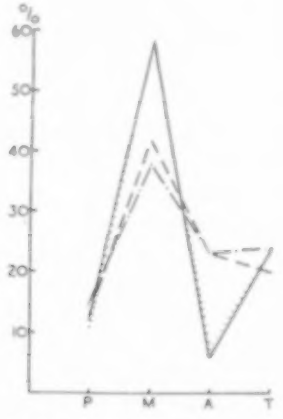

Fig. 1. Percentage distribution of mitotic phases in cultures of chick embryo osteoblasts: 15 min after application of aminopterin ————, and after 24 hr — — — —. A second application of aminopterin on the latter cultures. . . . . Saline control—·—· (N.B. aminopterin without cells retains its inhibitory power for 48 hr at 37 °C). P = prophase; M = metaphase; A = anaphase; T = telophase.

Methods

Cultures were grown in a medium containing 0·5 mg aminopterin per ml; after 48 hr of incubation, some were fixed (group II) or a small drop of a fresh solution of aminopterin (0·5 mg/ml) was deposited on the tissue for 15 min (group III). Control cultures, which had grown for 48 hr without aminopterin, were treated for 15 min with a drop of either Tyrode alone (group IV) or a fresh aminopterin solution (group I).

Results (*Fig.* 1)

In the cultures grown in the presence of aminopterin for 24–48 hr, the distribution of mitotic stages was now normal (42 per cent metaphases and 23 per cent anaphases)

as compared with controls grown for the same period without the inhibitor (38 per cent metaphases and 23 per cent anaphases). This result showed that the inhibition had been overcome. When the cultures which had grown for 48 hr in aminopterin were exposed for 15 min to a second application of aminopterin, however, they showed the typical arrest in metaphase with clumped chromosomes (58 per cent metaphases and 7 per cent anaphases) corresponding to the response obtained with the normal cultures exposed to aminopterin for the first time (58 per cent metaphase and 6 per cent anaphases) (Table 1).

TABLE 1. A SECOND APPLICATION OF AMINOPTERIN IS AS EFFECTIVE AS THE FIRST

(Chick osteoblasts, having inactivated aminopterin (II) in the course of 48 hr remain sensitive to a second application of the inhibitor (III). No "resistance" to aminopterin has developed. 48-hr-old cultures were used throughout. Groups (I) and (IV) were grown in normal medium. Groups II and III in the same medium + 0·5 mg aminopterin per ml.

	Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)	Number of mitoses
I. Aminopterin 15 min	12	58	6	24	491
II. Aminopterin 48 hr	13	42	23	22	588
III. Aminopterin 48 hr, followed by aminopterin 15 min	11	58	7	24	1009
IV. Tyrode control 15 min	15	38	23	24	584

Group III shows the same aminopterin effect caused by a second application as Group I, which has been exposed to aminopterin for 15 min only.
Group II is indistinguishable from the normal controls (Group IV).
The number of cultures used in the four groups were seven, four, nine and five, respectively.

These results indicated that the cells grown for 48 hr in aminopterin had overcome the metaphase block set by this compound, but remained as sensitive as before to a fresh application of the inhibitor. This ruled out the possibility that the cells used a metabolic path independent of folinic acid, and showed that they had not become resistant to aminopterin.

II. *The Inactivation of Aminopterin by Tissues*

Object of experiments

To test the ability of different types of cells to inactivate aminopterin.

Methods

The effect of various tissues taken from normal adult and embryonic chickens, from normal and leukaemic adult mice of the FAK_1 and AK strains, and from normal and leukaemic adults and children, on aminopterin was investigated.

Cell suspensions or finely cut pieces of tissue were incubated with aminopterin in a liquid medium at 37 °C under sterile conditions. The liquid media used were serum, a mixture of serum and Tyrode, or Tyrode alone. The experiments included the two controls previously mentioned, though occasionally in the case of human bone marrows not sufficient material was available for the tissue control. The concentration of aminopterin was usually 0·5 mg/ml, though in some of the experiments with human bone marrow the concentration was higher.

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Experiments were made to compare the effect of the cells of normal mouse lymph nodes (i.e. normal lymphocytes and lymphoblasts) on the one hand, and of leukaemic lymph nodes on the other. The leukaemia was of the acute lymphoblastic type. Only those nodes were selected which were almost replaced by leukaemic cells. Suspensions of normal or leukaemic lymphoid cells were prepared under sterile conditions as follows: Axillary, inguinal and mesenteric nodes were isolated and cut into small pieces with fine scissors; the tissue fragments were transferred to Tyrode with a pipette and the suspension was gently shaken, which caused most of the lymphoid cells to be washed out of the cellular reticulum. After standing for a few minutes, the small tissue fragments had sunk to the bottom, leaving a milky suspension of lymphocytes and lymphoblasts which was used for the experiment. Haematocrit determinations showed that these preparations contained about 25 mg of cells per ml. Batches of 2 and 4 ml of this type of suspension were incubated in small, flat-bottomed flasks for 24 hr at 37 °C, one of each group in Tyrode alone and the other in Tyrode containing 0·5 mg aminopterin per ml. After incubation the cell suspensions were centrifuged and the supernatants tested on tissue cultures in the way described.

Results

1. *Normal chicken tissue.* In addition to the tissues already investigated (Jacobson, 1954 *a*, *b*), fowl red blood cells and buffy coat containing a few red cells were found to inactivate aminopterin. After an extensive series of tests, beginning with 250 mg red cells per ml, progressively smaller quantities of red cells were used in these experiments, all of which showed that fowl erythrocytes inactivated aminopterin. It was found that 25 mg red blood cells per ml Tyrode converted 0·5 mg aminopterin to an inactive substance. The same quantity of red cells was capable of inactivating even 2 mg aminopterin per ml, and the same quantity of buffy coat collected after 30 min of centrifuging at 3000 rev/min had the same effect. When the supernatants were applied to test cultures for 15 min the results listed in Table 2 were obtained: experiments III and IV show clearly the inactivation of aminopterin.

These results also confirm the previous observation that chick serum has no effect on aminopterin. In view of the great capacity of fowl red cells to inactivate aminopterin this method was used extensively for the experiments to be described in a separate paper.[7]

2. *Mouse tissues.* (a) Normal liver. This tissue, finely minced, inactivated aminopterin within 24 hr, as was shown in the following experiment: 14·5 mg liver/ml, were incubated with 0·5 mg aminopterin/ml in a total volume of 12·8 ml Tyrode. The "tissue control" contained 16 mg liver/ml in a total volume of 14·6 ml Tyrode. The aminopterin control was 0·5 mg/ml in Tyrode. These three fluids were incubated for 24 hr and the supernatants tested for 15 min on the usual cultures. The results are summarized in Table 3 and show that aminopterin has lost its inhibitory power after incubation with liver. This capacity of the liver was of interest, as normal bone marrow (10 mg/ml) had previously been found unable to inactivate aminopterin.[5]

(b) Leukaemic cells from leukaemic lymph nodes. In previous experiments[4] it had been shown that mouse leukaemic cells, collected either from the area of implantation or the heavily infiltrated bone marrow, inactivated aminopterin, in contrast to the normal bone marrow which had no effect on the inhibitor. As normal mouse bone

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TABLE 2. THE INACTIVATION OF AMINOPTERIN BY RED CELLS AND THE BUFFY COAT OF FOWL'S BLOOD

(Distribution of mitotic phases in 48-hr cultures of chick osteoblasts, 15 min after the application of the following test solutions. Four cultures were used for each group.)

Test solutions	Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)	Number of mitoses
I. Aminopterin control 2 mg/ml Tyrode	11	73	2	14	654
II. Aminopterin control 2 mg/ml serum + Tyrode, equal parts	10	74	2	14	477
III. Aminopterin 2 mg/ml Tyrode + r.b.c. 25 mg	10	39	28	23	1063
IV. Aminopterin 2 mg/ml Tyrode + buffy coat 25 mg	9	42	28	21	347
V. R.B.C. control 25 mg/ml Tyrode (no aminopterin)	10	43	23	24	440
VI. Untreated cultures	13	41	27	19	470

Aminopterin has retained its inhibitory power after incubation for 24 hr with fowl serum (II) but has lost its inhibitory effect after incubation for 24 hr with fowl red cells (III) or buffy coat (IV).

TABLE 3. INACTIVATION OF AMINOPTERIN BY NORMAL MOUSE LIVER

(Distribution of mitotic phases in 48 hr cultures of osteoblasts, 15 min after the application of the test solutions.)

	Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)	Number of mitoses
Aminopterin control	9	74	2	15	340
Liver + aminopterin	7	52	19	22	601
Liver in Tyrode	7	51	20	22	558

Five, five and four cultures, respectively, were used for the three groups.
Aminopterin incubated with liver has lost its inhibitory power.

marrow contains probably no lymphoblasts and only a small percentage of lymphocytes (up to 10 per cent), it was decided to compare the inactivating capacities of normal and leukaemic cells from lymph nodes, as this would be a more significant experiment.

The control supernatants of the normal and of the leukaemic lymphoid cells incubated in Tyrode without aminopterin had no effect on cell division, as was to be expected, and the ratio of metaphases to anaphases was 46 per cent and 23 per cent in the test cultures (group II, Table 4). There was, however, a striking difference between the normal and the leukaemic cells in their action on aminopterin. The normal lymphocytes and lymphoblasts failed to inactivate this compound, which retained its

full inhibitory power after incubation with these cells; in the test cultures the metaphase count rose to 68 per cent and the anaphases count fell to 1 per cent (group III, Table 4). The leukaemic cells, on the other hand, converted the aminopterin into an

TABLE 4. INACTIVATION OF AMINOPTERIN BY LEUKAEMIC CELLS FROM LYMPHNODES OF MICE WITH ACUTE LYMPHOBLASTIC LEUKAEMIA (GROUP IV) AND INABILITY OF NORMAL LYMPHOID CELLS TO INACTIVATE AMINOPTERIN (GROUP III)

(Distribution of mitotic phases 15 min after application of the test solutions.)

	Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)	Number of mitoses
I. Aminopterin control (0·5 mg/ml)	10	70	1	19	297
II. Normal and leukaemic lymphoid cells (25 mg/ml)	9	46	23	22	235
III. Normal lymphoid cells (23 mg/ml) + aminopterin (0·5 mg/ml)	14	68	1	17	573
IV. Leukaemic lymphoid cells (8·2 mg/ml) + aminopterin (0·5 mg/ml)	11	46	20	23	478

Group III is indistinguishable from group I; in group IV no aminopterin effect can be discerned. In the four groups four, three, five and four cultures respectively were used.

inactive substance so that the supernatant had no inhibitory effect on cell division in the test cultures (group IV, Table 4).

3. *Human tissues.* (a) Normal blood. Aminopterin in concentrations of 0·5 or 1·0 mg/ml was incubated for 24 hr at 37 °C under sterile conditions with either whole blood (0·5–2·0 ml) or with the washed red cells from these volumes of blood, suspended in Tyrode's salt solution. The wet weight of red cells in the incubation fluids varied between 25 and 200 mg/ml. Serum or heparinized plasma (0·01 mg/ml) either undiluted or diluted with Tyrode, was also investigated for its effect on aminopterin (0·5 or 1·0 mg/ml). The blood of twenty-five normal humans was examined and the results are based on mitotic counts of 233 test cultures.

Plasma or serum, diluted or undiluted, had no effect on aminopterin which retained its full inhibitory action on cell division when applied to the test cultures. A total of sixty-one cultures were treated with aminopterin previously incubated with plasma or serum; in all cultures, metaphases were between 72 and 58 per cent and anaphases between 0 and 9 per cent among over 5000 mitoses; the results were indistinguishable from those obtained with aminopterin incubated in Tyrode (the aminopterin controls) and tested on twenty-six cultures. Serum, plasma or the supernatant or red cell suspensions in the absence of aminopterin, served as the "tissue" controls and had no effect on cell division in the test cultures (thirty-one cultures).

Whole blood or red cells resuspended in Tyrode, converted aminopterin into an inactive compound in twenty-one of twenty-five cases, In the twenty-one cases which

TABLE 5. FAILURE OF NORMAL HUMAN RED BLOOD CELLS TO INACTIVATE AMINOPTERIN

(In four of twenty-five cases aminopterin (1 mg) retained its inhibitory power after exposure to r.b.c. (400–450 mg) for 24 hr. Distribution of mitotic phases 15 min after application of the test solutions.)

	R.B.C. + aminopterin						Serum + aminopterin						Serum without aminopterin					
	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures
1	12	63	4	21	239	4	12	70	2	16	134	3						
2	12	72	0	16	218	4	13	71	0	16	472	4	17	41	20	22	592	4
3	11	61	7	21	290	4	14	69	4	13	251	4						
4_1	11	60	8	21	442	4	12	70	5	13	555	4	12	43	17	28	514	4
4_2	15	57	8	20	341	4	15	59	7	19	286	4						
4_3	11	57	8	24	523	4	15	62	7	16	245	4	13	43	22	22	283	4

In 4_2 and 4_3 aminopterin (1 mg) was exposed to 900 mg r.b.c. but retained most, if not all of its inhibitory action.

inactivated aminopterin the test cultures showed values well within the normal range, varying from 36–46 per cent metaphases and 18–28 per cent anaphases in eighty cultures with over 11,000 mitoses. However, in the four cases which failed to inactivate aminopterin (Table 5), the test cultures (twenty-four) had 60–72 per cent metaphases and 0–8 per cent anaphases, thus indicating an undiminished activity of the inhibitor. In all four cases at least 400–450 mg red cells failed to affect 1 mg aminopterin and in one of the four, during one of the re-tests with 900 mg red cells/ml, aminopterin retained most if not all of its inhibitory power (Table 5). No explanation can be offered for the difference between these four subjects and the twenty-one others all chosen at random.

(b) Lymphocytes. To determine whether the mature lymphocytes are responsible for the inactivation of aminopterin by human blood, three samples of 2 ml cerebrospinal fluid with high lymphocyte counts were incubated with 1 mg aminopterin. The fluid was sampled from two patients with meningitis (due to *Myco. tuberculosis* and *H. influenzae*) and one with cerebral degeneration; the content of lymphocytes was 1 mg, 0·5 mg and 0·2 mg per ml, respectively. Aminopterin was not inactivated by any of these, and test cultures showed the typical aminopterin effect. The ratios of meta- to ana-phases found in the three tests were: 71 per cent M / 2 per cent A, 70 per cent M / 2 per cent A, and 73 per cent M / 3 per cent A, respectively.

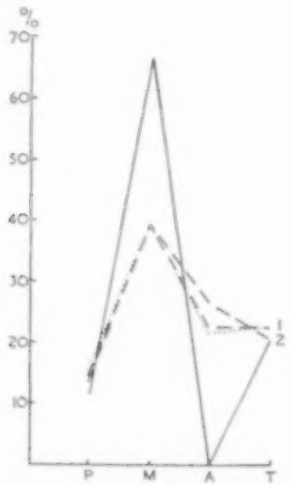

FIG. 2. Percentage distribution of mitotic phases in test cultures exposed for 15 min to: aminopterin ———; aminopterin previously incubated for 24 hr with the leukaemic bone marrow of case 1 — — —; aminopterin incubated previously for 24 hr with the leukaemic bone marrow of case 2 — —; serum control (of case 1) . . . . Abbreviations as in Fig. 1.

(c) Leukaemic bone marrow. Samples of bone marrow from eighteen children with acute leukaemia were incubated for 20–24 hr with 1 mg aminopterin. The amount of leukaemic cells in these samples varied widely as is usual in sampling human bone marrow; it ranged from 1 mg (three cases) to 110 mg (three cases of 100 mg or more), with an average of 21 mg leukaemic cells. In seventeen cases aminopterin was inactivated (see Fig. 2) and in only one case did it retain its inhibitory power (Table 6).

TABLE 6. THREE CONSECUTIVE MARROW SAMPLES OF A CHILD WITH ACUTE LEUKAEMIA (CASE 18)

(The first two, taken before a remission, failed to inactivate 1 mg aminopterin; the third sample was taken in remission, during aminopterin treatment, and there is an indication that aminopterin is now partially inactivated. Distribution of mitotic phases 15 min after application of the test fluids.)

	Total white cells (mg)	Leuk. cells (mg)	R.B.C. (mg)	Bone marrow + aminopterin						Serum + aminopterin						Serum without aminopterin					
				P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures
18_1	2	1·7	16	11	63	9	17	520	5							11	50	19	20	1005	4
18_2	2	1·3	50	11	64	9	16	432	5							10	50	20	20	315	2
18_3	2	0·1	20	10	53	15	22	849	4	12	64	5	19	409	3	9	47	24	20	370	3

The aminopterin inactivated by the seventeen marrows of leukaemic children was tested on sixty-six cultures with over 9000 cell divisions and was found to be unable to arrest mitoses in metaphase. The values obtained in the test cultures ranged between 40 and 48 per cent metaphases and between 17 and 27 per cent anaphases. Controls of aminopterin incubated with serum (plus Tyrode) from the samples retained their full inhibitory action on cell division in test cultures: 67–73 per cent metaphases and 0–1 per cent anaphases among 3000 mitoses. Serum plus Tyrode, without aminopterin, had no effect on cell division in the test cultures which showed a normal distribution pattern of the mitotic phases: 39–47 per cent metaphases and 18–24 per cent anaphases among over 4000 dividing cells. One of these seventeen cases may have been a borderline case of only partial inactivation of aminopterin (56 per cent M / 15 per cent A) by 22 mg leukaemic cells; however, a repeat marrow sample with only 7 mg leukaemic cells caused inactivation of aminopterin by the bone marrow cells. It is of interest that such small quantities of cells as used in these experiments were capable of converting 1 mg aminopterin into an inactive form. It is evident that the first two bone marrow samples of the eighteenth case (Table 6) failed to convert aminopterin during the first part of the disease and this patient responded subsequently to aminopterin treatment. The third sample taken towards the end of the remission, indicates that part of the aminopterin had been inactivated by incubation with marrow cells; the compound had lost some of its inhibitory qualities: 53 per cent M / 15 per cent A, when compared with the aminopterin controls 64 per cent M / 5 per cent A or the serum controls 47 per cent M / 24 per cent A.

Thus seventeen of the eighteen bone marrows of children with acute leukaemia had the capacity to convert aminopterin into an inactive compound. In the eighteenth case the capacity to inactivate aminopterin developed in the course of the disease.

DISCUSSION

(1) *The aminopterin effect on cell division*

Folic acid antagonists cause arrest of dividing cells in the metaphase stage. This effect has been observed in chick embryo fibroblasts and osteoblasts growing in tissue culture, where the cells can be exposed directly to the inhibitor, and also in the bone marrow of leukaemic children, and in normal and leukaemic marrow of mice and in their intestinal epithelium, whether the antagonist is given parenterally or by mouth. The lesions which these compounds can cause in the human intestinal mucosa are so strikingly similar to those produced experimentally in the mouse, that the human intestinal mucous membrane with its numerous cell divisions appears to be affected in the same way. Thus the arrest of cell division in metaphase by folic acid antagonists can be viewed as a general phenomenon.

Though many other quite unrelated compounds can cause a similar arrest of cell division in metaphase, the particular interest of the inhibition caused by folic acid antagonists lies in the fact that it can be prevented completely and over a considerable range of concentrations by the simultaneous application of folinic acid. The crucial experiment can only be performed on cells growing *in vitro*, where the conversion of folic to folinic acid can be precluded. In this way it was possible to demonstrate that folinic, and not folic acid, functions for a period of a few minutes during the step from meta- to ana-phase. The inhibition analysis, by exposing cells simultaneously

and directly to both the inhibitory analogue and the physiological compound, established that folinic acid has an important part to play in cell division. It appears to be the first instance of a defined chemical substance to which such a specific function during mitosis can be allocated.

For this reason, and in view of the clinical use of the folic acid antagonists in the treatment of acute leukaemias in childhood, it was relevant to investigate the so-called resistance to these compounds. They exert a striking effect initially, particularly in tissue cultures where mitotic arrest occurs within a few minutes, but finally become ineffective after longer exposure to all leukaemic cells and some normal cells.

Since cells growing *in vitro* show an easily observable response on exposure to folic acid antagonists, they provided a suitable test system for the activity of these compounds. Test cultures treated with aminopterin or other folic acid antagonists for 15 min, show not only an accumulation of metaphases and a decrease of anaphases, but also a severe clumping of the metaphase chromosomes in contrast to the control cultures treated with saline, serum, tissue fluids or inactivated aminopterin. Because of these distinctive features, and since no overlap occurred in the percentage values of the mitotic pattern of cultures treated with folic acid antagonists and those treated with the inactivated compound, a subsidiary reliance on statistical evaluation of the results was not required.

As to the specificity of the test the following points are to be mentioned: (1) Folinic acid prevents the inhibition caused by aminopterin over a range of concentrations. Folic acid fails to do this. (2) Aminopterin after exposure to some tissues retains its inhibitory qualities, but after exposure to other tissues it loses its inhibitory action. (3) Inactivated aminopterin, after treatment with mild acid, for example, regains its capacity to inhibit cell division in the test cultures. These three points provided the basis for considering the test system of cells growing *in vitro* as satisfactory and sufficiently specific.

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2. *The inactivation of folic acid antagonists*

It was surprising to find that some normal human red blood cells can convert aminopterin (and A-methopterin as found in experiments to be reported later) into an inactive form, and that this quality of normal human red blood cells was not shared by all subjects. Attempts to correlate these observations with other, probably enzymic qualities of the red cells should be an interesting task. It was equally surprising that rather small quantities of leukaemic human bone marrow were very effective in inactivating aminopterin and A-methopterin. It was unfortunately not possible at this stage of the investigation to correlate the *in vitro* inactivation of aminopterin with the clinical response, but this will be attempted. The very small quantities of human bone marrow usually available did not allow the separation of white and red cells and it is notorious that attempts to increase the yield of leukaemic marrow usually lead to dilution with blood; but the proportion of the two types of cells in some of the marrows indicates that the leukaemic cells are at least as effective as the red cells in converting aminopterin into an inactive form. The experiments with mouse leukaemic tissue allowed a better separation of the two types of cells. Whenever the preparations of leukaemic cells were checked for contamination with red cells this was found to be very low. The cell-free fluids, whether serum, plasma or tissue fluids,

were always found to be without any effect on aminopterin. This has been found previously for a Tyrode extract of crushed chick embryo osteoblasts and was repeatedly confirmed with the supernatant of human leukaemic marrow, which by necessity must always contain some dissolved material from cellular débris, especially after a 24 hr incubation period.

Acknowledgements—The writers gratefully acknowledge the interest and help of Dr. H. B. Fell, F.R.S., Director of the Strangeways Research Laboratory, and thank Mrs. June Buscall for her skilful assistance throughout this work.

The generous supply of folic acid antagonists by Dr. J. Smith, Jr., Director of the Research Division of the American Cyanamid Co. is acknowledged with gratitude.

The expenses of this work were defrayed by the Medical Research Council.

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Vol. 5 1960

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THE NATURE OF AMINOPTERIN INACTIVATED BY NORMAL AND LEUKAEMIC TISSUES

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(*Received* 27 *May* 1960)

Abstract—(1) Inactivated aminopterin can be reactivated by a brief exposure, at room temperature, to either mild acid or alkali.

(2) The u.v. absorption spectrum of aminopterin and its inactive form are indistinguishable.

(3) A-methopterin, the N_{10}-methyl analogue of aminopterin, is also inactivated, but amino-an-fol, a weak folic acid antagonist which differs from aminopterin by a terminal aspartic instead of a glutamic acid, resists inactivation.

(4) The *in vitro* inactivation of aminopterin does not occur in the presence of relatively high concentrations of glutamate or *p*-aminobenzoylglutamate.

(5) The Mg salt of inactivated aminopterin indicates that a free carboxylic acid group has been lost in the course of inactivation.

(6) In view of the evidence listed under points (1)—(5) the tentative hypothesis is proposed that inactivation of aminopterin is caused by a ring closure of the terminal glutamic acid.

(7) The possible implications of these findings for the treatment of acute leukaemias are discussed.

INTRODUCTION

IN A previous paper[1] experiments were described which showed that the folic acid antagonist aminopterin (4-aminopteroylglutamic acid) loses its capacity to arrest cell division at the metaphase stage after incubation with a variety of normal tissues or leukaemic cells from mice or children with acute leukaemia.

Folic acid antagonists are used in the investigation of cellular processes and as therapeutic agents. They act by interfering with the function of folinic acid, which is essential for all cell divisions and for the *de novo* synthesis of purines and thymine in the two daughter cells after completion of mitosis. Furthermore, they are capable of inducing temporary remissions in a certain percentage of children with acute leukaemia. It was therefore of interest to conduct experiments which may throw light on the nature of the inactivation of folic acid antagonists, especially as the majority of children with acute leukaemia fail to respond to these compounds and those who initially responded become eventually resistant to treatment with folic acid antagonists. The results of the investigations reported here suggest that the inactivation of aminopterin may be due to a ring formation of the terminal glutamic acid of the molecule.

MATERIAL AND METHODS

Aminopterin dissolved in Tyrode's salt solution was inactivated by incubating it with either human or fowl red blood cells or with leukaemic cells of mice with an acute lymphoblastic leukaemia. The concentrations of aminopterin varied between 0·5 and 2·5 mg/ml. The quantities of cells varied between 10 and 50 mg/ml. The total volumes of the incubation mixtures ranged from 2 to 20 ml. The fluids were kept in a shallow layer for 20–24 hr at 37 °C. They were then centrifuged and the supernatant, containing the incubated aminopterin was either tested immediately or stored at 3 °C for 1 or 2 days. All solutions and materials were prepared throughout under strictly sterile conditions.

The standard procedure for testing, whether aminopterin had retained its ability to arrest mitoses in metaphase, or whether it had lost its inhibitory power, were performed on hanging drop tissue cultures, 48 hr old, of chick embryo frontal bones. These cultures provided abundant supply of cells in division. The test fluid (0·01–0·02 ml) was placed on the cultures for 15 min. They were then fixed in methanol and stained with May–Grunwald's and Giemsa's dyes. Further details of the procedure are given in a previous paper (Jacobson and Cathie, 1960).

Dividing cells were counted and classified as prophases (P), metaphases (M), anaphases (A) and telophases (T) and recorded as percentage of all dividing cells.

In each experiment control cultures were counted, so that each experiment had its own internal standards for comparison. Untreated cultures, and those exposed for 15 min to supernatants of cells incubated without aminopterin, showed a normal distribution pattern of mitotic phases (about 40–45 per cent metaphases and about 20–25 per cent anaphases). Similar values were found in cultures exposed for 15 min to fully inactivated aminopterin. In contrast, test cultures exposed for the same engtth of time to aminopterin showed a striking arrest in metaphases (about 60–70 per cent) with severe clumping of the chromosomes, and very few anaphases (0–10 per cent). Thus by comparing the metaphase (M) and anaphase (A) ratios in the tables the reader can appraise the essential part of the information. Special technical procedures are given in the corresponding sections.

RESULTS

Seeking information about the chemical nature of the inactivated aminopterin, a number of tests were undertaken which can be grouped into two classes: those that would indicate whether a partial breakdown of the molecule had taken place, and those that would show whether there had been a transformation without involving a katabolic process. Over fifty preparations of inactivated aminopterin were used for this purpose.

1. *Tests to determine whether aminopterin breaks down during inactivation*

All of the investigations in this group gave negative results.

(a) *Tests for deamination.* When 10 mg of aminopterin, inactivated by incubation with fowl or human red blood cells or mouse leukaemic cells, were placed in a Conway diffusion unit, no ammonia was liberated, and similar tests with Nessler's reagent were also negative. No sign of urea formation could be detected during the inactivation of 10 mg aminopterin with fowl or human red blood cells, when the solution containing the inactivated compound was treated with urease, followed by Nessler's reagent.

(b) *Tests for liberation of free pteridine,* p-*aminobenzoic acid,* p-*aminobenzoylglutamic acid, and glutamic acid in the course of inactivation of aminopterin.* After aminopterin was incubated with either human or fowl red blood cells or mouse

leukaemic cells, neither the ultra-violet abosrption sectrum nor the emission spectrum of the inactivated form showed any evidence for the liberation of a pteridine, nor could this be demonstrated by extraction with *n*-butanol into which a free pteridine might have been concentrated. The Bratton and Marshall[2] test with naphthylethylenediamine for aromatic amines was negative; there was thus no indication of the presence of either free *p*-aminobenzoic acid or free *p*-aminobenzoylglutamic acid. No free amino acid could be demonstrated with ninhydrin, so that the setting free of the glutamic acid part of the compound could be excluded.

(c) *Experiments to determine whether the inactivation of aminopterin was caused by acetylation or a similar acylation.* Inactivated aminopterin was treated with a mixture of phosphoric acid and phosphorus pentachloride, to form an acylchloride; subsequent treatment with *n*-butanol, to form butylacetate or other butyl esters, failed to show the presence of acetate or other volatile fatty acids. Distillation of the acid hydrolysate of the inactivated compound did not reveal any volatile acids. These findings exluded the possibility that acetylation of aminopterin or a similar process was the cause of inactivation.

(d) *Inconclusive attempts to distinguish aminopterin from its inactive form chromatographically.* Four different solvents were used: 3% NH_4Cl, *n*-butanol + 5N-acetic acid + water (8 : 1 : 1), 5% sodium citrate saturated with *iso*amyl alcohol, and 70% *iso*propanol in water.

In the first two solvents, both aminopterin and inactivated aminopterin gave long streaks instead of defined spots. The front of the streaks were at the same positions for both forms. This indicated that both compounds may have undergone some alteration in the course of the procedure. The third solvent (5% sodium citrate saturated with *iso*amyl alcohol) did not distinguish between the active and the inactive form (both R_f 0·20). The u.v. absorbing area of a paper chromatogram obtained from 0·5 mg aminopterin and 0·5 mg inactivated aminopterin was eluted with 1 ml Tyrode and the eluates were tested in the usual way on four cultures each: the eluate of the aminopterin chromatogram inhibited mitoses (P 9 per cent, M 67 per cent, A 6 per cent, T 18 per cent in 418 dividing cells); the eluate of the inactivated aminopterin did not inhibit mitoses (P 8 per cent, M 49 per cent, A 20 per cent, T 23 per cent in 414 dividing cells).

The two derivatives of aminopterin: N_5-formyl-, 5:6-, 7:8-tetrahydroaminopterin and N_{10}-formylaminopterin, kindly supplied by Dr. Slavikova,[8] could be excluded as products of inactivation as their R_f-values, under these conditions, were 0·60 and 0·52.

2. *Tests to determine whether a molecular "transformation" without breakdown of aminopterin occurs in the course of inactivation*

(a) *The ultra-violet absorption spectra of aminopterin and its inactivated form.* The u.v. absorption spectra of the two compounds in Tyrode's solution were indistinguishable (Fig. 1). This observation established that in the pteridine and benzene moieties of the two compounds no substantial alteration could have occurred. Any change in the system of conjugated double bonds, such as hydrogenation, or substitutions on certain positions of the pteridine or benzene rings, would have caused characteristic changes in the ultra-violet absorption spectrum.

(b) *The effect of mild acid or alkaline hydrolysis on inactivated aminopterin.* Brief exposure of inactivated aminopterin to very mild acid or alkali at room temperature led to re-activation of the compound, restoring its inhibitory power on cell divisions in the test cultures.

In view of the lability of aminopterin itself on treatment with acid, a series of experiments were undertaken to determine at what degree of acidity aminopterin would not be destroyed. When 0·5 mg aminopterin in 1 ml plasma + Tyrode (1 : 1) was mixed with 1 ml of 0·3N HCl at room temperature for 3 min and was then

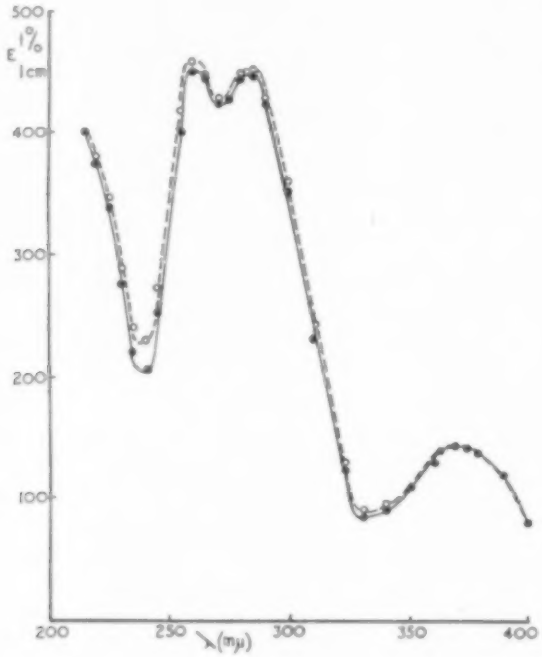

FIG. 1. U.V. absorption spectrum of aminopterin (●——●) and inactivated aminopterin (○ — — ○)

neutralized with 0·3N NaOH (this required 0.75 ml), it failed to arrest mitoses in the test cultures (Table 1, group 1). By a stepwise reduction of the amount of acid added to aminopterin solutions (0·5 mg/ml plasma + Tyrode, equal parts), it was found (Table 1) that the addition of 0·1 ml of 0·3N HCl per ml at room temperature did not diminish the inhibitory activity of aminopterin during the first 3 min (Table 1, group 4), though some reduction might occur during 30 min (Table 1, group 5). It was therefore assumed that in an attempt to reactivate the inactivated aminopterin it would be safe to expose the inactive form for short periods to 0·05 ml of 0·3N HCl per ml.

The inactivated compound used throughout these experiments was prepared by incubation of aminopterin in Tyrode with either human or fowl red blood cells or mouse leukaemic cells. If 0·05 ml of a 0·3N hydrochloric acid was added at room temperature for each ml of the solution containing 0·5 mg inactivated aminopterin

TABLE 1. DESTRUCTION OF AMINOPTERIN BY ACID AT ROOM TEMPERATURE

(0·5 mg aminopterin per ml plasma and Tyrode equal parts. The neutralized solutions are applied to two or more test cultures for 15 min.)

	Vol. of 0·3 N HCl added to each ml of aminopterin sol. (ml)	Time of exposure to acid (min)	Vol. of 0·3 N NaOH required for neutralization (ml)	% Ratio of meta- to ana-phase in test cultures (%)
(1)	1	3	0·75	45–20
(2)	0·5	3	0·34	47–22
(3)	0·2	3	0·16	53–13
(4)	0·1	3	0·05	71–2
(5)	0·1	30	0·06	63–1
(6)	0·05	20	0·02	70–1

Under the first three conditions aminopterin has lost all or most (3) of its inhibitory action. Under the last three conditions aminopterin has retained all or nearly all (5) of its inhibitory action.

per ml and this was then carefully neutralized with 0·3N sodium hydroxide to pH 7·2–7·3 with the aid of a micropipette, the inhibitory action of the inactivated compound was restored, provided the exposure to acid did not exceed 15 min. The course of the reactivation is illustrated by the following experiment (Fig. 2): to the solution containing the inactivated aminopterin, acid was added in the quantity described above.

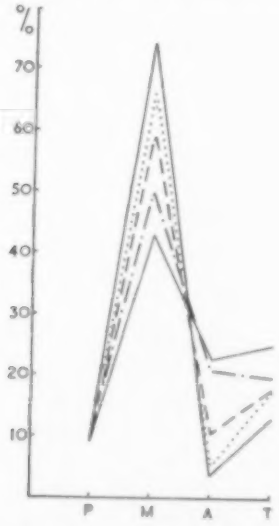

FIG. 2. Reactivation of inactive aminopterin by acid treatment. Distribution of mitotic phases in test cultures after 15 min exposure to: inactivated aminopterin ——— (metaphases, 43 per cent); inactivated aminopterin after treatment with 0·05 ml 0·3 N HCl/ml for 3 min — — — —; for 5 min curve not drawn, metaphases 64 per cent, marked X; for 15 min . . . . . ; for 30 min—·—·. Aminopterin control ——— (metaphases, 74 per cent). Abbreviations: P, prophase; M, metaphase; A, anaphase; T, telophase.

Aliquots were neutralized after 3, 5, 15 and 30 min of exposure to the acid. These four samples were applied to groups of test cultures. The following served as controls (1) inactivated aminopterin, not exposed to acid, (2) aminopterin in Tyrode, exposed to acid for corresponding periods, and (3) as the tissue control, the supernatant of red cells incubated without aminopterin (Table 2).

It can be seen that exposure of the inactive compound to acid for 3,5 and 15 min led to increasing inhibitory action on cell division. After 30 min exposure to acid,

TABLE 2. REACTIVATION OF INACTIVE AMINOPTERIN BY ACID TREATMENT

(The inhibitory action is restored increasingly up to 15 min exposure to acid. The neutralized solutions were applied to test cultures for 15 min. The mitotic ratios were as shown in the Table.)

The controls	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures
(1) 0·5 mg aminopterin per ml serum + Tyrode (1 : 1)	9	74	4	13	196	3
(2) 0·5 aminopterin + 100 mg r.b.c./ml incub. 24 hr	9	43	23	25	246	3
(3) 100 mg r.b.c./ml Tyrode, incub. 24 hr	9	44	24	23	230	2
Acid treated solution (2) = inactivated aminopterin + 0·05 ml 0·3 N HCl/ml.						
Neutralized after 3 min	9	60	11	20	127	2
Neutralized after 5 min	9	64	6	21	204	2
Neutralized after 15 min	10	66	6	18	246	2
Neutralized after 30 min	9	52	21	18	260	2

Neutralization required 0·02 ml 0·3 N NaOH/ml in each case. For acid treatment of aminopterin (incubated without r.b.c.) see Table 1.

The inactivated aminopterin (solution 2) has regained most of its inhibitory power after 15 min exposure to acid. P = prophase, M = metaphase, A = anaphase, T = telophase.

however, the inhibitory power of the compound was much reduced. The interpretation of this observation was made possible by the second control group in which the active, unaltered aminopterin was treated with the same amount of acid for corresponding periods. It was found that under these conditions aminopterin itself was partially destroyed when exposed to 0·3N hydrochloric acid for 30 min; longer exposure, e.g. 1 hr, or the use of 1 ml 0·3N HCl per ml of the aminopterin solution led to complete loss of the inhibitory action (Table 1) possibly by deamination. It can be assumed, therefore, that exposure to 0·3N HCl, 0·05 ml/ml for periods of not more than 15 min converts the inactive into the active form, but beyond this period the active form is destroyed at a rate faster than that of the reactivation process. Thus, extending the exposure to acid beyond a point when a maximum has been reached, leads to an irreversible destruction of aminopterin.

When inactivated aminopterin in Tyrode (2·5 mg/ml) was exposed to 0·3N NaOH (0·1 ml/ml aminopterin solution) for 15 min at room temperature and then carefully neutralized with 0·3N HCl, some of its inhibitory action was restored (Table 3). The inactivated aminopterin produced the mitotic ratios M, 46 per cent, A, 24 per cent, but after it had been exposed to alkali for 15 min it gave the ratios: M, 61 per cent, A, 6 per cent, and after 30 min of exposure, M, 53 per cent, A, 12 per cent. It may be concluded that alkali, like acid, reactivates the compound, but that prolonged exposure to alkali destroys some of the aminopterin formed by alkaline hydrolysis.

TABLE 3. REACTIVATION OF INACTIVE AMINOPTERIN BY TREATMENT WITH ALKALI

(The inhibitory action was restored increasingly up to 20 min exposure to alkali (solution 3). The neutralized solutions were applied to test cultures for 15 min. The mitotic ratios were as shown in the Table.)

Control	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures
(1) Inactivated aminopterin (aminopterin 2·5 mg/ml) (+ r.b.c. 50 mg/ml)	10	46	24	20	679	7
Inactivated aminopterin (= solution 1) + 0·1 ml 0·3 N NaOH/ml						
(2) Neutralized after 15 min	11	61	6	22	340	2
(3) Neutralized after 20 min	10	70	4	16	969	4
(4) Neutralized after 30 min	13	53	12	22	391	4

30 min of exposure to alkali lead already to a partial destruction of aminopterin, the inhibitory power is decreased. (N.B. aminopterin itself exposed to alkali for 1 hr loses all its inhibitory action). Abbreviations as in Table 2.

Hence 30 min of treatment with NaOH results in less inhibitory activity than 15 min. This is also borne out by an experiment in which aminopterin itself was treated for 1 hr with the same amount of NaOH, when nearly all its inhibitory power was destroyed (M, 48 per cent, A, 17 per cent).

In view of the reactivation of inactivated aminopterin by very mild treatment with acid or alkali at room temperature, it was tentatively assumed that a ring closure of the terminal glutamic acid might be the process underlying the inactivation. The validity of this assumption was tested by the following three experiments (sections c, d and e).

(c) *Inactivation of A-methopterin by red blood cells and failure of red blood cells to inactivate amino-an-fol.* If inactivation is caused by ring closure, then A-methopterin (4-amino-N_{10}-methylpteroylglutamic acid), which is an effective folic acid antagonist, should be inactivated under the same conditions as aminopterin, since both compounds have a terminal glutamic acid. On the other hand, amino-an-fol (4-aminopteroyl-aspartic acid), which is a rather weak folic acid antagonist, should retain its inhibitory power, since it carries a terminal aspartic acid, instead of a glutamic acid. Aspartic acid could not be readily cyclized, as its carbon chain would be too short (see Fig. 5). A-methopterin inhibited cell divisions in cultures of embryonic chick osteoblasts and

in human bone marrow,[3] and the chick cells growing in cultures were able to overcome this inhibition within 24 hr.

In the present experiments, when 1 mg A-methopterin was incubated with 20 mg human red blood cells it lost its effect on cell division: M, 48 per cent, A, 21 per cent (among 893 mitoses of four cultures) (Fig. 3). The same amount of A-methopterin incubated with 18 mg r.b.c. + 1 mg leukaemic cells of a child with acute leukaemia in relapse after treatment with this compound, also lost its inhibitory action on cell division: M, 45 per cent, A, 23 per cent (among 439 mitoses in four cultures). Finally 10 mg red cells plus less than 1 mg leukaemic cells of the bone marrow from a child

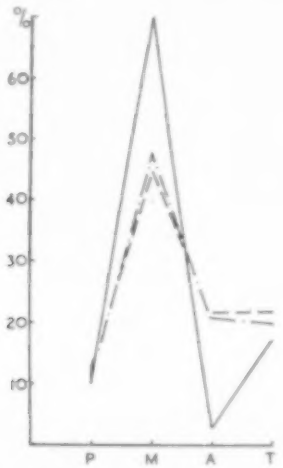

FIG. 3. Inactivation of A-methopterin by red blood cells. Distribution of mitotic phases in test cultures 15 min after exposure to: A-methopterin (2·5 mg/ml)———; A-methopterin (2·5 mg/ml), previously incubated for 24 hr with red blood cells (20 mg/ml.) —·—·—·; serum controls — — —. Abbreviations as in Fig. 2.

with acute leukaemia inactivated 1 mg A-methopterin: M, 46 per cent, A, 22 per cent (among 181 mitoses in three cultures). Thus A-methopterin is inactivated by the same tissues as aminopterin.

In contrast, when 4-aminopteroylaspartic acid (amino-an-fol) was incubated with human red cells (of the same blood as that used for the experiment with A-methopterin and with the same quantities) it did not lose its inhibitory action on cell division in test cultures: M, 65 per cent, A, 5 per cent (among 949 mitoses in seven cultures): the control, amino-an-fol incubated for 24 hr without red cells produced a similar mitotic ratio: M, 70 per cent, A, 4 per cent, (among 1108 mitoses in seven cultures) (Fig. 4).

(d) *Magnesium salts of inactivated aminopterin and of aminopterin.*[7] A ring closure of the terminal glutamic acid of aminopterin would entail a loss of the γ-carboxylic acid. This could be demonstrated by the formation of an insoluble salt with, e.g. Mg^{2+} or Ba^{2+}: either of the bivalent cations would combine with one molecule of active, and two molecules of the inactivated aminopterin. Thus the weight of Ba^{2+} or Mg^{2+} in the salt of the inactivated compound would be about half that in the aminopterin salt.

(1) Inactivated aminopterin was prepared as follows: each of two flasks received 20 ml Tyrode in which were dissolved 40 mg aminopterin and to which were added 400 mg packed red cells. The flasks were incubated for 24 hr, under sterile conditions at 37 °C, the contents were centrifuged and the clear yellow supernatant containing the incubated aminopterin was tested on cultures. It had no inhibitory action on cell division (M, 48 per cent, A, 21 per cent among 551 mitoses); untreated control cultures showed M, 47 per cent, A, 22 per cent (among 526 mitoses). The aminopterin, therefore, had been inactivated. The solution was deproteinized by shaking with chloroform, centrifuged and the clear yellow solution pipetted off. This process was repeated four times, until no precipitate with chloroform was formed. The inactivated aminopterin was stored overnight at 0 °C. A yellow, microcrystalline precipitate was collected by centrifuging at 3 °C and twice washed in a small volume of ice-cold,

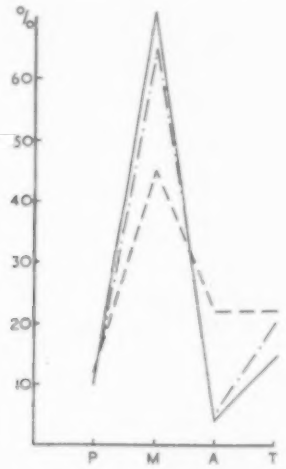

FIG. 4. Failure of red blood cells to inactivate amino-an-fol. Distribution of mitotic phases in test cultures 15 min after exposure to: amino-an-fol 2·5 mg/ml inc. 24 hr. ———; amino-an-fol, 2·5 mg/ml previously incubated for 24 hr with red blood cells 20 mg/ml, inc. 24 hr —·—·—·; serum controls — — —. Abbreviations as in Fig. 2.

glass-distilled water; care was taken to insure that the material stayed at temperatures between 0 ° and 3 °C. After the washed suspension of the precipitate had been centrifuged at 3 °C, the water was drained off and the fine yellow crystals were dried *in vacuo* over P_2O_5. To the yellow supernatant still containing a substantial part of the inactivated aminopterin, were added 3 vols. of ethanol, and this was placed at − 23 °C overnight. A further microcrystalline precipitate was collected, washed, treated in the same way as the first precipitate and pooled with it. The dried, inactivated aminopterin was retested at this stage on dividing cells in cultures: it had remained inactive throughout these procedures, M, 47 per cent, A, 19 per cent (among 594 mitoses). The magnesium salt was prepared as follows. The remainder of the crystalline inactivated aminopterin (34·0 mg) was dissolved in 17 ml 2 per cent sodium acetate, $MgCl_2$ (20 mg anhydrous, dried *in vacuo*) was added. Some precipitate appeared already at room temperature. The flasks were stored at 0 °C overnight, and the considerably increased precipitate was collected by centrifuging at 3 °C; it was washed in

a small volume of ice-cold, glass-distilled water and centrifuged in the cold, after which the water was drained off and the precipitate dried *in vacuo* over P_2O_5. To the supernatant, containing the inactivated aminopterin and magnesium chloride, were added 3 vols of ethanol; this was stored at − 23 °C overnight and the second precipitate of fine yellow crystals was collected, treated as the first precipitate and pooled with it; 29·60 mg of the inactivated aminopterin-Mg-compound was ashed. The weight of the ash was 1·50 mg as MgO, which corresponds to a Mg^{2+} content of inactivated aminopterin of 3·0 per cent. The theoretical value for inactivated aminopterin + $Mg^{2+}/2$ (m.w. 433·3) would be 2·8 per cent.

(2) The following controls were prepared: (a) Aminopterin was incubated in Tyrode without red blood cells, and treated in the same way as the inactivated aminopterin, except that it was shaken with chloroform once only. When tested on cultures it was found to have retained its inhibitory capacity at the end of these procedures, and gave the mitotic ratios: M, 63 per cent, A, 5 per cent (among 440 mitoses). When ashed as a free compound no measureable or visible ash could be detected. (b) The Mg salt of aminopterin was prepared in the same way as described above for the inactivated aminopterin. 52·14 mg of the Mg salt of aminopterin, when ashed, yielded an ash weight of 4·55 mg, which, as MgO, corresponds to a Mg^{2+} content of 5·26 per cent for aminopterin-Mg compound (m.w. 462·5). The theoretical value of the Mg^{2+} content is 5·26 per cent.

The following "blanks" were prepared: Tyrode solution, when shaken with chloroform and stored at 0 °C, did not form a precipitate; it remained clear when 3 vols. ethanol were added and when this mixture was stored at − 23 °C. A solution of 2% sodium acetate (1 vol.) + ethanol (3 vols.) + $MgCl_2$ (11·3 mg/10 ml 2% sodium acetate) gave no precipitate on storage at − 23 °C for several days. The $MgCl_2$ used gave the correct theoretical value of MgO when ashed.

Thus the difference in the Mg content of the magnesium salts of aminopterin (5·26 per cent) and of inactivated aminopterin (3·0 per cent) would not be inconsistent with the assumption that in the course of inactivation one free carboxylic acid is blocked, possibly by a ring closure of the terminal glutamic acid (Fig. 5).

(c) *The protective action of glutamate and* p-*aminobenzoylglutamate* (*p.a.b. glut.*) *against inactivation of aminopterin* in vitro. If inactivation of aminopterin involved an alteration of the terminal glutamic acid, it would be of interest to investigate the effect of relatively high concentrations of glutamate and p.a.b. glut. on this process. Loading the incubation mixture of aminopterin and blood cells with either glutamate or *p*-aminobenzoylglutamate might saturate the inactivating enzyme system to such an extent that the aminopterin molecules may be protected and thus not so readily inactivated.

The following technical points had to be considered before setting up the experiment. The addition of glutamic and *p*-aminobenzoylgluatmic acid to the incubation mixture would raise the tonicity of the solution and lower its pH unduly. This was an important point in view of the instability of aminopterin in acid solutions. It was found that 10 mg glutamic acid or 10 mg p.a.b. glut., dissolved in 0·2 ml 3·5% $Na_2HPO_4 + 12H_2O$ and brought to pH 6·5 by the addition of 0·03 ml 1·5N NaOH, could be added to 1·77 ml Tyrode to make 2 ml total volume, without affecting the

tonicity unduly. The final pH values of the incubation mixtures, containing the cells, were between 7·0–7·3.

Besides the standard aminopterin control and the tissue control (i.e. these two incubated separately and alone) the following three additional control systems had to be set up: (1) aminopterin incubated in the presence of glutamate or p.a.b. glut.; (2) leukaemic cells incubated in the presence of these two substances without

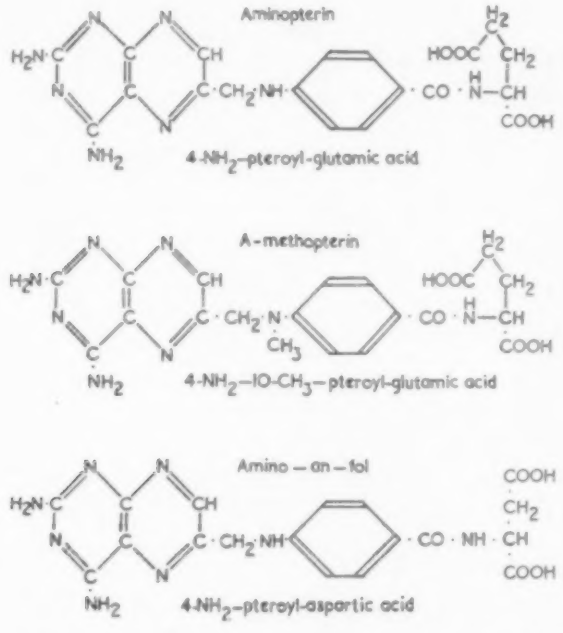

FIG. 5. The formulae of the three folic acid antagonists drawn to illustrate the hypothesis of inactivation by ring closure of the terminal glutamic acid in the case of aminopterin and A-methopterin, and to illustrate the failure to inactivate amino-an-fol.

aminopterin; (3) leukaemic cells and aminopterin incubated together in the presence of additional phosphate. This was made up of a mixture of Na_2HPO_4 and NaH_2PO_4 to give the equivalent values of added phosphate, as used in the solution for the test substances. All these controls were satisfactory: the aminopterin control (without leukaemic cells) retained its inhibitory action on dividing cells, after it had been incubated in the presence of glutamate or p.a.b. glut., and the leukaemic cells inactivated aminopterin in the presence of additional phosphate.

When aminopterin was incubated with leukaemic cells in the presence of relatively high concentrations of glutamate or p.a.b. glut. it retained its capacity to arrest mitosis in the test cultures (Figs. 6 and 7), while without these two compounds aminopterin was inactivated by the leukaemic cells. These results are summarized in Table 4, and show that either glutamate or p.a.b. glut. can protect aminopterin from being inactivated by leukaemic cells. The effect of other amino acids has not yet been tried.

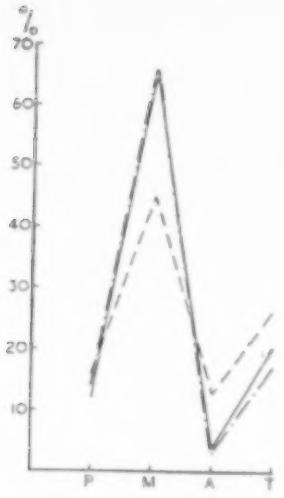

FIG. 6. Protection of aminopterin by glutamate against inactivation by leukaemic cells. Distribution of mitotic phases in test cultures 15 min after application of the following solutions previously incubated for 24 hr: aminopterin 1:500 + glutamate 1:200 ———; leukaemic cells + aminopterin 1:500 + glutamate 1:200 —·—·—·; leukaemic cells + glutamate 1:200 — — —. Abbreviations as in Fig. 2. All solutions in Tyrode for 24 hr at 37 °C.

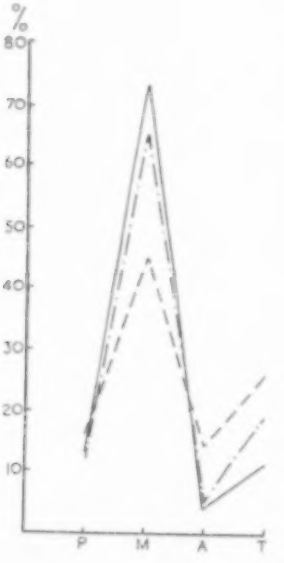

FIG. 7. Protection of aminopterin by *p*-aminobenzoylglutamate (p.a.b. glut) against inactivation by leukaemic cells.

Distribution of mitotic phases in test cultures 15 min after application of the following solutions previously incubated for 24 hr: aminopterin 1:500 + p.a.b. glut. 1:200 ———; leukaemic cells + aminopterin 1:500 + p.a.b. glut. 1:200 —·—·—·; leukaemic cells + p.a.b. glut. 1:200 — — —. Abbreviations as in Fig. 2.

TABLE 4. PROTECTION OF AMINOPTERIN BY GLUTAMATE OR *p*-AMINOBENZOYLGLUTAMATE (P.A.B. GLUT.) AGAINST THE INACTIVATION BY LEUKAEMIC CELLS

Distribution of mitotic phases in the test cultures 15 min. after application of the solutions.

	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures
(1) Tissue controls:						
(a) leuk. cells + glutamate	20	48	14	18	124	3
(b) leuk. cells + p.a.b. glut.	16	45	13	26	232	3
(2) Aminopterin controls:						
(a) aminopterin + glutamate	12	65	3	20	991	6
(b) aminopterin + p.a.b. glut.	13	73	3	11	173	2
(3) Leuk. cells + aminopterin	11	50	19	20	982	8
(4) Leuk. cells + aminopterin + glutamate	14	66	3	17	1687	16
(5) Leuk. cells + aminopterin + p.a.b. glut.	12	65	4	19	1653	15

Groups (4) and (5) show the protection of aminopterin against inactivation by leukaemic cells in the presence of glutamate or p.a.b. glut. The test cultures of these two groups show the typical aminopterin effect, i.e. arrest of mitoses in metaphase. All fluids were supplemented with phosphate and were incubated for 24 hr. The concentrations used were: aminopterin 2·0 or 2·5 mg/ml, glutamate or p.a.b. glut. 5 mg/ml, mouse leukaemic cells 25 mg/ml. Abbreviations as in Table 2.

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DISCUSSION

A variety of normal tissues convert aminopterin into an inactive substance in the course of 20–24 hr, so that cells exposed to this form of aminopterin go through cell division unimpaired and can make full use of their endogenous folinic acid. Cells which have inactivated aminopterin can be arrested again in mitosis by a second application of aminopterin. This indicates that the cell still requires folinic acid for mitotic division.[1, 3–5, 7] The inactivation of aminopterin leads to an apparent resistance of cells to the action of the inhibitor. This aspect is of importance in interpreting biological and biochemical observations when the function of folinic acid and its derivatives is investigated by means of folic acid antagonists over a period of several hours or days. The case of leukaemic cells which are either resistant to folic acid antagonists like aminopterin or A-methopterin from the onset or become so in the course of treatment, makes the question of the mechanism by which this resistance is achieved particularly relevant.

As to the nature of the inactivation of aminopterin by red blood cells and leukaemic cells it must be stressed that the hypothesis advanced in this paper cannot be more than a working hypothesis, that inactivation is brought about by a ring closure of the terminal glutamic acid of the aminopterin molecule. Further work will show whether this hypothesis is tenable or not, but no evidence could be obtained for any of the well-known mechanisms of detoxication.

The following facts concerning the process of inactivation can be stated and are consistent with the assumption that the site of inactivation may be localized in the glutamic acid moiety of the aminopterin molecule. (1) The inactive form is labile and

can be reactivated by brief exposure to either mild acid or alkali at room temperature. (2) the u.v. absorption spectrum of aminopterin and its inactive form cannot be distinguished. (3) There is a significant difference in the Mg content of the magnesium salts of aminopterin and its inactive form. (4) The 4-aminopteroylaspartic acid analogue is not attacked in the same way as the two other antagonists investigated. In view of this observation, it may be worth re-investigating the clinical effectiveness of this compound which was tried about 10 years ago[6] but was superseded by the two which were more effective per mg. (5) Since glutamate and *p*-aminobenzoylglutamate can protect aminopterin *in vitro* against inactivation by mouse leukaemic cells during 24 hr of incubation, experiments will now be undertaken to find out whether a similar protective mechanism can be invoked in the treatment of acute leukaemias with aminopterin or A-methopterin.

Acknowledgements—The writer gratefully acknowledges the interest and help of Dr. H. B. Fell, F.R.S., Director of the Strangeways Research Laboratory and thanks Mrs. June Buscall for her skilful assistance throughout this work. The generous supply of folic acid antagonists by Dr. J. Smith, Jr., Director of the Research Division of the American Cyanamid Co. is acknowledged with gratitude, as is the supply of the two formyl derivatives of aminopterin by Dr. Slavíková.[8] The expenses of this work were defrayed by the Medical Research Council.

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SYNTHESIS OF 5-CHLORODEOXYURIDINE AND A COMPARATIVE STUDY OF 5-HALODEOXYURIDINES IN *E. COLI**

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(*Received January*, 1960)

Abstract—5-Chlorodeoxyuridine (ClUDR) was synthesized for the purpose of comparing its inhibitory properties with 5-fluorodeoxyuridine (FUDR), 5-bromodeoxyuridine (BrUDR) and 5-iododeoxyuridine (IUDR). With *Escherichia coli* K 12 as the test organism, it was found that FUDR was about 4000 times more effective than ClUDR which, in turn, was nine times more effective than BrUDR for inhibiting growth of the bacteria to 50 per cent of a control. IUDR was not inhibitory to growth of *E. coli* K 12. Low concentrations of ClUDR, BrUDR or IUDR strongly potentiated the inhibitory effect of FUDR in *E. coli* over a 200- to 300-fold concentration range by limiting the utilization of thymidylate for DNA synthesis. Stimulation of growth with higher concentrations of ClUDR, BrUDR or IUDR in the presence of FUDR indicates that these halogen derivatives serve as thymidine substitutes for DNA synthesis.

INTRODUCTION

AN INTERESTING feature of the inhibitory effects of 5-bromodeoxyuridine (BrUDR),§ 5-fluorodeoxyuridine (FUDR) and 5-iododeoxyuridine (IUDR) on nucleic acid metabolism is that the nature of the halogen in the 5-position of the pyrimidine influences markedly the effectiveness and type of inhibition produced by the deoxynucleoside derivatives. FUDR inhibits DNA synthesis by blocking the methylation of deoxyuridylate to thymidylate,[1, 2] whereas BrUDR[3, 4] and IUDR[5] inhibit incorporation of thymine compounds into DNA. In addition, 5-bromouracil,[6–8] 5-chlorouracil[8] and 5-iodouracil[6, 8] replace thymine of DNA, while 5-fluorouracil,[1, 9, 10] and to a lesser extent 5-chlorouracil,[11] are incorporated in place of uracil into RNA.

5-Chlorodeoxyuridine (ClUDR) was prepared to make possible additional comparisons of the inhibitory effects of 5-halodeoxyuridine derivatives. ClUDR has been identified previously as a constituent of DNA from bacteria grown in the presence of 5-chlorouracil.[12, 13]

Escherichia coli K 12 was chosen as a test organism for the comparative studies because FUDR, ClUDR or BrUDR inhibit growth of this strain which does not require exogenous pyrimidines for growth. Therefore inhibitory effects of the halodeoxyuridine compounds may be assumed to be the result of interference with

* Supported by a grant from the U.S. Public Health Service No. C-2373 (C3).

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‡ Taken in part from the thesis submitted by Bessie Huang to the Faculty of the Graduate School of Southern California, in partial fulfillment of the requirements for the Master of Science Degree in Biochemistry and Nutrition. Supported by a training grant by the USPHS.

§ The abbreviations used are: BrUDR, 5-bromo-2′-deoxyuridine; ClUDR, 5-chloro-2′-deoxyuridine; FUDR, 5-fluoro-2′-deoxyuridine; IUDR, 5-iodo-2′-deoxyuridine; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

intermediates arising *de novo* rather than with intermediates of "salvage" pathways of synthesis, such as the conversion of pyridimidine nucleosides to the corresponding 5′-phosphates.

MATERIALS AND METHODS

Materials

Deoxyuridine, deoxycytidine, uridine, cytidine, orotic acid, thymidine, thymidine-5′-phosphate and IUDR were purchased from the California Corporation for Biochemical Research, Los Angeles. FUDR was obtained through the courtesy of the Cancer Chemotherapy National Service Center, Bethesda, Maryland. BrUDR was synthesized by methods previously described.[14] ClUDR was prepared as described below.

5-*Chlorodeoxyuridine*

Powdered deoxyuridine (5·25 g, 0·023 mole) was dissolved in 300 ml of glacial acetic acid at room temperature. A 10% excess of dry chlorine in 200 ml of cold anhydrous carbon tetrachloride was added and the solution allowed to stand overnight. After removal of the solvents by lyophilization, the residue was deacetylated in 400 ml of anhydrous methanol containing from 10 to 15% anhydrous ammonia. Hydrolysis was complete upon standing at room temperature for 3 days. The volume was reduced to 30 ml and crystallization was induced by addition of ethyl acetate until the solution became cloudy. The crystalline product which appeared upon standing was recrystallized from absolute ethanol. The white crystals, 1·95 g (30 per cent yield), melted at 178–179·5 °C (uncorrected). The λ of maximum and minimum absorption was 278 mμ and 239 mμ, respectively, at pH 7 (a_M max., 10·06 $\times$ 10^3).

Anal. Calcd. for $C_9H_{11}O_5N_2Cl$: C, 41·15; H, 4·22; N, 10·67. Found: C, 41·05; H, 4·06; N, 10·91.

Assay Methods

The halodeoxyuridine compounds were assayed with *E. coli* K 12 in a glucose salts medium using methods previously described.[15] The tubes were incubated at 37 °C for 18 hr, at which time growth of the controls was maximal. Growth was measured as turbidity in a Klett–Summerson photoelectric colorimeter (filter no. 66). All determinations were run in duplicate or triplicate.

RESULTS AND DISCUSSION

The growth response of *E. coli* K 12 to increasing concentrations of FUDR, ClUDR, BrUDR and IUDR is summarized in Fig. 1. Comparison of the concentrations of the analogs which limit growth to 50 per cent of a control reveals that FUDR is about 4000 times more inhibitory than ClUDR which, in turn, is about nine times more active than BrUDR. IUDR was not inhibitory at concentrations up to 2000 μM. Complete repression of growth for an 18-hr period was produced by 0·08 μM FUDR or 1600 μM ClUDR, but 2600 μM BrUDR caused only partial inhibition. Growth was completely suppressed for an indefinite period by 3 μM FUDR, whereas 2600 μM ClUDR did not inhibit growth during longer incubation periods. 5-Chlorouracil, 5-bromouracil or 5-iodouracil were not inhibitory.

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Several pyrimidine compounds known to participate in the biosynthesis of nucleic acids were tested for their ability to reverse the growth inhibition of *E. coli* K 12 by ClUDR or FUDR (Figs. 2–4). Such inhibition reversal studies are often not informative in determining the nature of inhibitory action. However, since FUDR, IUDR and BrUDR have been studied extensively and known to have inhibitory actions at specific

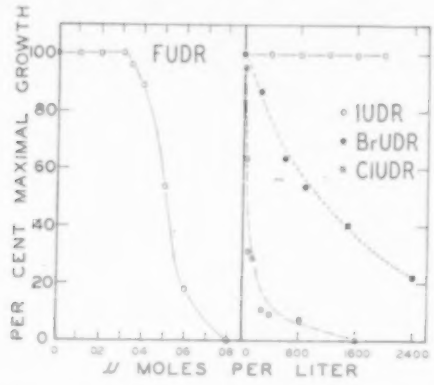

FIG. 1. Comparison of the inhibitory effect of 5-fluorodeoxyuridine (FUDR), 5-iododeoxyuridine (IUDR), 5-bromodeoxyuridine (BrUDR) and 5-chlorodeoxyuridine (ClUDR) on the growth of *E. coli* K 12. Incubated at 37 °C for 18 hr in a glucose–salts medium.

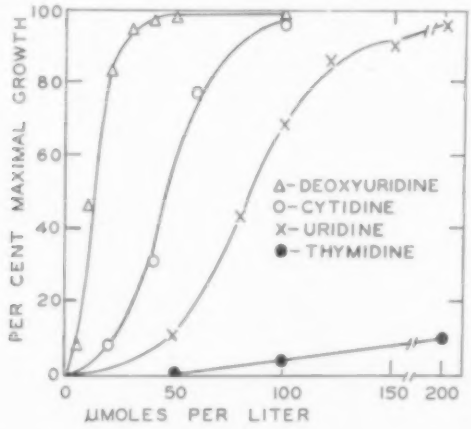

FIG. 2. Comparison of the effectiveness of various pyrimidine compounds in reversing the growth of *E. coli* K 12 in the presence of 0·5 μM 5-fluorodeoxyuridine. Incubated at 37 °C for 18 hr in a glucose–salts medium.

metabolic sites, it was expected that interpretation of reversal data in the presence of ClUDR would be more specific than is usually possible.

It is apparent from the data of Figs. 2–4 that cytosine and uracil ribonucleosides or deoxyribonucleosides are effective to a variable extent in reversing the inhibitory effects of ClUDR or FUDR. No valid interpretations can be made from the fact that differences were observed in the effectiveness of these nucleosides in reversing inhibition because facile interconversions are possible. For example, cytosine compounds

may be converted to uracil compounds by deaminases, and deoxynucleosides may be precursors of ribonucleotides by a prior cleavage to uracil with deoxynucleoside phosphorylase. The relative rates of these reactions *in vivo* are not known. In addition, it is probable that the halodeoxyuridine compounds inhibit to a variable extent the

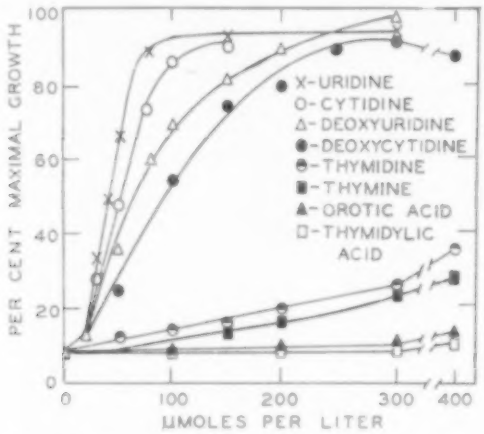

FIG. 3. Comparison of the effectiveness of pyrimidine compounds for reversing the growth of *E. coli* K 12 in the presence of 800 μM 5-chlorodeoxyuridine. Incubated at 37 °C for 18 hr in a glucose–salts medium.

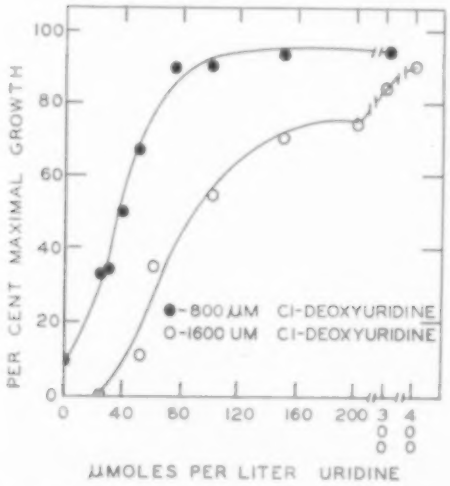

FIG. 4. Reversal of *E. coli* K 12 growth by uridine at two concentrations of 5-chlorodeoxyuridine. Incubated at 37 °C for 18 hr in glucose–salts medium.

conversion of the reversing agents to compounds of the nucleotide pool. At a concentration of FUDR which is about six times the minimum concentration for complete inhibition, thymidine is a relatively ineffective reversing agent (Fig. 2). It is apparent from Fig. 3 that thymine, thymidine and thymidylic acid are ineffective reversing agents at a concentration of ClUDR which does not produce complete inhibition. Deoxyuridine, uridine, deoxycytidine and cytidine reverse the inhibition of

ClUDR to approximately that of control growth (Fig. 3), but as shown in Fig. 4 reversal with uridine is not competitive.

Thymine compounds do not undergo the facile interconversions mentioned above. For this reason, and since FUDR,[1, 2] BrUDR[3, 4] and IUDR[5] are known to inhibit metabolism of thymine compounds, the effectiveness of thymidine for reversing inhibitions produced by halodeoxyuridine compounds was of interest. In the presence of 0·061 μM FUDR, which allows 2 per cent of control growth, thymidine is an effective reversing agent, but at higher concentrations of FUDR (0·5 μM) thymidine produced only a slight reversal effect (Fig. 5). These results support previous data[1, 2]

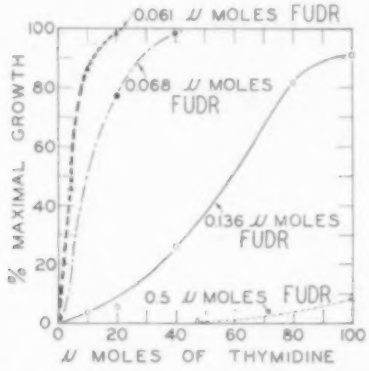

FIG. 5. Reversal of *E. coli* K 12 growth by thymidine in the presence of various concentrations of 5-fluorodeoxyuridine (FUDR). Incubated at 37 °C for 18 hr in glucose–salts medium.

indicating that the primary effect of FUDR on *E. coli* growth is the inhibition of thymidylate synthetase. The additional inhibitory effects, which probably are the result of interference with RNA metabolism by prior cleavage to fluorouracil,[2] can be overcome with either uracil or cytosine nucleosides (Fig. 2).

Fig. 6 summarizes the effectiveness of thymidine as a reversal agent in the presence of ClUDR. At 800 μM ClUDR, thymidine had no effect. Only partial reversal was produced by thymidine in the presence of 200 μM ClUDR which allowed about 30 per cent of control growth. From this it may be concluded that, if ClUDR inhibits thymidylate synthetase or thymidylic acid utilization, there are additional sites of inhibition at this concentration. The lack of a good growth response to thymidine in the presence of uridine and 800 μM ClUDR (Fig. 6) substantiates the above conclusion.

Since bromouracil, chlorouracil and iodouracil are incorporated into DNA in place of thymine under conditions of thymine deficiency,[12] it was of interest to determine the effect of the corresponding nucleoside derivatives on growth of *E. coli* K 12 in the presence of a low concentration of FUDR which specifically limits thymidylate synthesis (Fig. 5). At these conditions it is reasonable to expect that BrUDR, ClUDR or IUDR would be incorporated into DNA to a greater extent than in the presence of an uninhibited supply of endogenous thymidine phosphates. Furthermore, since FUDR limits thymidylate synthesis,[1, 2] and BrUDR[3, 4] or IUDR[5] inhibits thymidylate utilization, it seemed logical to predict that a combination of FUDR with BrUDR or IUDR would act synergistically as growth inhibitors. It was therefore of interest to compare the effects of various concentrations of ClUDR, IUDR and BrUDR on

growth and viability of *E. coli* in the presence of a low concentration of FUDR which limits thymidylate formation selectively[1, 2] (Figs. 7 and 8).

Fig. 7 summarizes the data obtained from the use of a combination of ClUDR and FUDR. Almost identical results were obtained by substituting BrUDR or IUDR for ClUDR (Fig. 8). At a concentration of FUDR (0·034 μM), which allows 97 per cent maximal growth, 0·03 μM to 10 μM ClUDR, IUDR or BrUDR inhibited

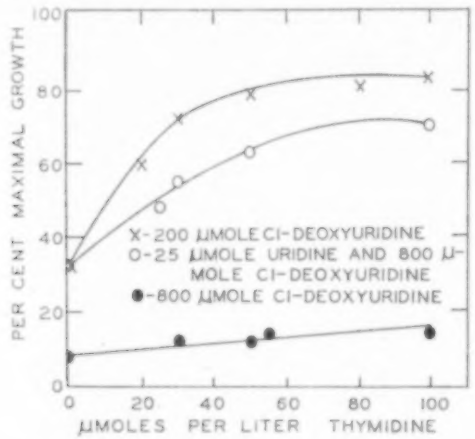

Fig. 6. Reversal of *E. coli* K 12 growth by thymidine in the presence of 5-chlorodeoxyuridine. Incubated at 37 °C for 18 hr in glucose–salts medium.

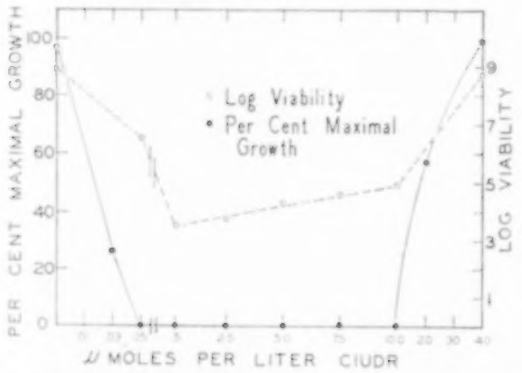

Fig. 7. Effect of various concentrations of 5-chlorodeoxyuridine (ClUDR) in the presence of 0·034 μM 5-fluorodeoxyuridine (FUDR) on growth and viability of *E. Coli* K 12. Incubated at 37 °C for 18 hr in glucose–salts medium.

growth completely and cell viability was reduced to about 1 per cent of the inoculum. Thus, although IUDR alone does not inhibit growth of *E. coli* K 12 at concentrations up to 2000 μM, it is a very potent inhibitor in the presence of FUDR which allows 97 per cent control growth. Similarly, ClUDR or BrUDR completely inhibit growth over a 200- to 300-fold concentration range (Figs. 7 and 8).

One μM thymidine or 10 μM thymine completely reversed the inhibitory effect of the combined halonucleosides. Viability and turbidity were also restored to

control growth by concentrations of ClUDR, BrUDR or IUDR above 10 μM. Thus, ClUDR, BrUDR or IUDR at low concentrations in combination with FUDR are potent inhibitors of growth and viability over a 200- to 300-fold concentration range, whereas higher concentrations of the same analogs reverse this effect (Figs. 7 and 8).

The synergistic effect on growth (Fig. 8) is consistent with activities of FUDR,[1, 2] BrUDR[3, 4] and IUDR,[5] which block sequential reactions of thymine nucleotide metabolism. BrUDR or IUDR, which are known to inhibit thymidylate utilization, are relatively poor inhibitors of *E. coli* K 12 growth when they are not used in combination with FUDR (Fig. 1). This ineffectiveness may be due to a rapid *de novo* synthesis

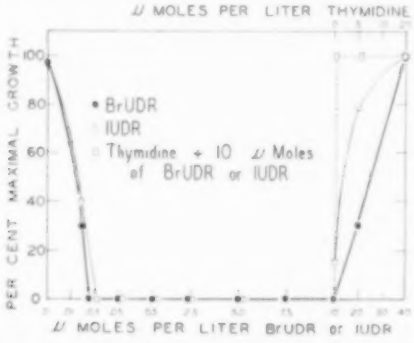

FIG. 8. Effect of various concentrations of 5-bromodeoxyuridine (BrUDR) or 5-iododeoxyuridine (IUDR) in the presence of 0·034 μM 5-fluorodeoxyuridine (FUDR) on growth of *E. coli* K 12. Incubated at 37 °C for 18 hr in glucose–salts medium.

of thymidylate. However, when thymidylate is limited by a concentration of FUDR which allows 97 per cent control growth, BrUDR or IUDR are potent inhibitors. It may be concluded that the combination of BrUDR and FUDR or IUDR and FUDR specifically limits the availability of thymine nucleotides for DNA synthesis since thymine or thymidine reverse the inhibitory effect completely.

The effects of ClUDR (Fig. 7) are almost identical to those produced by BrUDR or IUDR (Fig. 8). By analogous reasoning it may be concluded that ClUDR also inhibits utilization of thymidylate. From Fig. 3 it is apparent that ClUDR causes additional inhibitory effects which are reversed with uracil or cytosine nucleosides but not thymidine. These effects are not produced by a prior cleavage of ClUDR to 5-chlorouracil, since the latter compound, in contrast to 5-fluorouracil, does not inhibit *E. coli* K 12.

The observation that higher concentrations of ClUDR, BrUDR or IUDR (Fig. 7 and 8) circumvent both inhibitory effects and produce growth, suggests that these 5-halopyrimidine compounds substitute for thymine nucleotides in DNA synthesis. If this interpretation is correct, it may be concluded that substitution of the 5-halopyrimidines for thymine in DNA does not have a permanent lethal effect on the viability of a substantial population of the cells. This conclusion is consistent with the data of Zamenhof and Griboff[7] who found that from 17 to 19 per cent incorporation of 5-bromouracil into *E. coli* can occur without affecting the speed of cell division. A variable tolerance of tumour cells to replacement of thymine by 5-bromouracil has been demonstrated by Hakala[16] who found that the replacement limit in HeLa

cells is about 50 per cent and in mouse fibroblasts greater than 80 per cent. The similar effectiveness of ClUDR, BrUDR and IUDR in replacing the thymine requirement for growth suggests that chlorouracil, iodouracil and bromouracil are comparable in serving as non-lethal substitutes for DNA thymine.

It is unlikely that conditions (Figs. 7 and 8) are ideal for continued incorporation of unnatural bases into DNA during the entire 18-hr incubation period. As the number of cells increase, the conversion rate of nucleoside derivatives to free bases by nucleoside phosphorylase increases. Thus, as growth proceeds, it would be expected that the concentration of FUDR would drop[2] to a level which does not inhibit thymidylate formation. As endogenous thymidylate becomes available, incorporation of ClUDR, BrUDR and IUDR would be expected to decrease. This does not detract from the conclusion that growth is probably stimulated (Figs. 7 and 8) by incorporation of the halonucleosides into DNA in place of thymidylate during the first few hours of incubation.

It is probable[1, 2] that FUDR owes its anti-tumor activity to the fact that it produces cell death because of its primary action on thymidylate synthesis, thus producing a chemically-induced "thymidineless death" of rapidly dividing cells.[17] The present data show that in *E. coli* K 12 a relatively narrow concentration range of FUDR is effective for limiting thymidylate synthesis, without at the same time inducing other inhibitory mechanisms (Fig. 5). A thymidine-less condition is produced by a low concentration of FUDR in combination with ClUDR, BrUDR or BrUDR over a wide range of the latter compounds. This combination of inhibitors completely inhibits *E. coli* multiplication over 200- to 300-fold concentration range without inhibiting reactions other than those involving thymidylate formation or utilization. Under these conditions, DNA synthesis is limited specifically by an inadequate supply of thymine compounds and cell death as well as growth inhibition occurs (Fig. 7). At higher levels of ClUDR, BrUDR or IUDR in combination with FUDR, an entirely different effect may occur; namely cell death by incorporation of ClUDR, IUDR, or BrUDR into DNA.[16] These conditions offer interesting possibilities for chemotherapy investigations.

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SHORT COMMUNICATIONS

Effect of 1-aminocyclopentanecarboxylic acid on amino acid utilization in various bacterial test systems

(*Received* 8 *July* 1960)

1-AMINOCYCLOPENTANECARBOXYLIC acid has received considerable attention because of its ability to inhibit the growth of mouse sarcoma-180,[1] carcinoma-755 [1] and Novikoff rat hepatoma.[2] It was submitted to the Cancer Chemotherapy National Service Center by Dr. C. I. Noll of Pennsylvania State University for testing and was assigned the Code Number NSC-1026.[3] Though clinical trials with NSC-1026 were disappointing, the possibility existed of finding new and closely related compounds with improved effectiveness. Recently a number of structures closely related to NSC-1026 have been synthesized and tested against carcinoma-755, sarcoma-180 and leukemia-1210.[4] Almost without exception these derivatives were found to be ineffective. The specificity of the parent compound posed a problem of its mechanism of action.

Because of structural similarity between natural amino acids and NSC-1026, it was tested for the ability to interfere with amino acid utilization of 17 amino acids in a number of bacterial test systems. The amino acids studied were those commonly found on analysis of normal mouse and tumor tissues.[5]

Several bacterial test systems were employed,[6–10] each of which had a specific requirement for the amino acid against which NSC-1026 was tested. Table 1 presents the bacteria used, a reference to the basal medium, the maximum ratio tested of "antagonist" to amino acid, and the effects noted.

The bacterial cultures were provided a complete nutrient medium with sufficient amounts of the amino acid under test to allow approximately one-half maximum growth. A growth antagonist would reduce the growth rate below the half-maximal level if it interfered with the utilization of the particular amino acid in question. If growth exceeded the half-maximal level in the presence of the antagonist

TABLE 1. EFFECT OF 1-AMINOCYCLOPENTANECARBOXYLIC ACID ON THE UTILIZATION OF SEVENTEEN AMINO ACIDS BY BACTERIA

Test organism	Amino acid	Maximum weight ratio of drug to amino acid	Effect on utilization of amino acid	Reference to method and medium used
Lactobacillus delbrueckii LD5	L-phenylalanine	160 : 1	None	(8)
Lactobacillus delbrueckii LD5	L-aspartic	48 : 1	None	(6)
Leuconostoc mesenteroides P-60	L-aspartic	1000 : 1	None	(7)
Streptococcus faecalis R	L-isoleucine	500 : 1	None	(8)
Streptococcus faecalis R	L-valine	500 : 1	None	(8)
Streptococcus faecalis R	L-tyrosine	600 : 1	None	(9)
Streptococcus faecalis R	L-histidine	320 : 1	None	(8)
Streptococcus faecalis R	L-arginine	240 : 1	None	(8)
Streptococcus faecalis R	L-threonine	200 : 1	None	(8)
Streptococcus faecalis R	L-methionine	240 : 1	None	(8)
Streptococcus faecalis R	L-tryptophane	2000 : 1	None	(8)
Lactobacillus arabinosus 17-5	L-leucine	160 : 1	None	(8)
Lactobacillus arabinosus 17-5	L-glutamic	120 : 1	stim. 25%	(10)
Lactobacillus arabinosus 17-5	L-cystine	850 : 1	None	(10)
Lactobacillus arabinosus 17-5	L-serine	200 : 1	None	(7)
Leuconostoc mesenteroides P-60	L-lysine	200 : 1	None	(7)
Leuconostoc mesenteroides P-60	L-proline	200 : 1	None	(7)
Leuconostoc mesenteroides P-60	L-glycine	300 : 1	stim. 20%	(7)

it would be presumed that the antagonist was being metabolized in place of the missing amino acid. Growth was measured by titration of the acid formed in the culture.

An examination of the results in column 4 of Table 1 shows that under the test conditions used, NSC-1026 did not function as an antagonist of any of the 17 amino acids. In the case of *Lactobacillus arabinosus* 17-5 with L-glutamic acid, and *Leuconostoc mesenteroides* P-60 with glycine, there was evidence of some utilization of NSC-1026.

Ross[3] stated "the assumption could be made that NSC-1026 would function as an amino acid antagonist since it can be looked upon as an amino acid lacking the usual α-hydrogen atom". This hypothesis is not substantiated by the data presented in this report.

Acknowledgements—This work was aided in part by research grant CY-2479 from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service and, in part, by the Midwest Research Institute.

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The inhibition of gamma aminobutyric-alpha-ketoglutaric acid transaminase *in vitro* and *in vivo* by amino-oxyacetic acid

(*Received* 15 *July* 1960)

THERE is abundant evidence (Roberts and Frankel, 1950; Awapara, *et al.*, 1950; and Roberts, 1956) that γ-aminobutyric acid (GABA) is found in high concentrations in the central nervous system. However, administration of GABA in large doses does not result in increased concentrations of this amino acid in brain, presumably because of its failure to cross the blood-brain barrier (Van Gelder and Elliot, 1958). Baxter and Roberts (1959) demonstrated that inhibition of γ-aminobutyric acid-α-ketoglutaric acid transaminase occurred in rats after administration of suitable doses of hydroxylamine, with concomitant increases of 100 per cent in brain GABA concentrations. This report is concerned with aminoöxyacetic acid (AOAA), a more effective inhibitor of this transaminase.

A transaminase preparation isolated from *E. coli* ATCC-26 was used for testing compounds as potential inhibitors using the following conditions: 40 μ moles of GABA, 40 μ moles of α-ketoglutaric acid (KGA), 50 μ moles of borate buffer, pH 8·2, enzyme sufficient to form from 3 to 4 μ moles of glutamate per hr, and water to 1·5 ml. Analysis for glutamic acid was carried out as described by Baxter and Roberts (1958). Under these conditions, AOAA inhibited the enzyme 100 per cent at a concentration of $3{\cdot}3 \times 10^{-4}$M and 40 per cent at $3{\cdot}3 \times 10^{-6}$M. Further studies with the bacterial preparation revealed that the inhibition was competitive for both substrates of the enzyme. When tested with a transaminase preparation from brain which, in contrast to the bacterial enzyme, requires supplementation with pyridoxal phosphate (Baxter and Roberts, 1958), a 92 per cent inhibition was observed at 1×10^{-5}M.

Jakoby and Scott (1959) described a preparation of adaptive enzymes from *Pseudomonas fluorescens* ATCC-13, 430 grown on a medium rich in pyrollidine which, in addition to GABA transminase, also has succinic semialdehyde dehydrogenase. This partially purified preparation could be used to assay either GABA or KGA, spectrophotometrically, by measurement of TPNH. An analogous system was found in *E. coli* ATCC-26 grown in nutrient broth (Difco) without special addition of substrates. An isolation procedure was developed for these enzymes which results in a preparation containing from 60 to 70 per cent of the initial activity of sonicates with a 1·5- to 2-fold purification. This preparation was used for all determinations of GABA in brain, as follows: to a Beckman DU cuvette were added 5·0 μ moles of 2-mercapto-ethanol, 6·0 μ moles of α-ketoglutaric acid, 600 μ moles of Tris buffer, pH 8·35, 1·0 mg of TPN, from 250 to 300 units of enzyme, and water to a final volume of 3·0 ml. An enzyme unit is defined as that amount of enzyme which will induce a change of 0·001 per min in the optical density, when assayed with 6·0 μ moles of GABA, in addition to the above components.

The rate of reaction was measured at 1-min intervals for 5 min at 340 m μ. The reading taken at zero-time was subtracted from the reading at 5 min in order to obtain the extent of TPNH formation. Standard curves in the range of from 0·1 to 0·6 μ moles of GABA were linear and the error on known standards was less than 3·0 per cent. Brain samples were prepared for analysis in a manner similar to that described by Roberts and Frankel (1950). The frozen brains were blended in 10 volumes of ethanol–water (75 : 25), and an aliquot of the supernatant fluid from this homogenate was dried *in vacuo* and made up in 0·1 volume of the aqueous ethanol for assay. Of the concentrated extract, 0·05 ml usually were taken for analysis.

By this means it was demonstrated that AOAA, administered in appropriate dosage to rats, mice, cats, dogs, and guinea pigs, elevated brain GABA levels 4- to 5-fold. The peak levels of GABA occurred 6 hr after AOAA administration. Thereafter, the GABA levels declined, but in most species above-normal levels were observed even 24 hr after administration of the inhibitor. As the GABA levels increased after AOAA administration, definite depression of the central nervous system ensued. This effect is under further study. The GABA levels were confirmed by paper chromatography.

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Potentiation of carcinostasis by combinations of thioguanine and 6-mercaptopurine*

(*Received* 22 *August* 1960)

THE carcinostatic purine analogs 6-mercaptopurine and thioguanine are closely related in structure, and were at first thought to have similar mechanisms of action.[1] However, it has been shown by Greenlees and LePage[2] and Sartorelli *et al.*[3] that these drugs differ in their effects on purine metabolism in ascites tumors. In addition, their toxic effects are manifested in different tissues.[4] This report presents further evidence of the differences between 6-mercaptopurine and thioguanine by showing that combinations of these drugs produce a potentiation of the carcinostatic action. Furthermore, a tumor subline developed for resistance to thioguanine is only partially cross-resistant to 6-mercaptopurine.

* This work was supported by Contract No. SA-43-ph-3068 with the Cancer Chemotherapy National Service Center, National Institutes of Health, and by grants from the U.S. Public Health Service (CY-4551) and the Gustavus and Louise Pfeiffer Research Foundation.

Female Swiss mice, 25–30 g, were inoculated with 2×10^6 cells of the Ehrlich ascites carcinoma or a thioguanine-resistant subline of this tumor which has been described previously.[3] The drugs were administered intraperitoneally. Therapy was initiated 24 hr after tumor implantation and daily injections were continued for six days. All data are averages of results from two experiments.

The results of treatment of Ehrlich ascites carcinoma with eight combinations of thioguanine and 6-mercaptopurine are presented in Table 1A. Several combinations prolonged the average survival

TABLE 1. TREATMENT OF EHRLICH ASCITES TUMOR LINES WITH COMBINATIONS OF THIOGUANINE AND 6-MERCAPTOPURINE

Dose (mg/kg) Thioguanine	6-mercaptopurine	Average survival time (days)	50-day survivors/ total no. of animals	Average weight change (g)
(A) Ehrlich ascites				
0	0	10·6 ± 2·0*	0/15	+1·5
0	30	18·8 ± 1·8	0/15	+0·8
0	15	16·0 ± 2·5	0/15	+3·0
0	7·5	12·6 ± 0·9	0/15	+2·6
0	3·75	11·6 ± 3·0	0/15	+1·4
1	0	20·4 ± 1·9	0/15	+4·0
0·5	0	18·6 ± 2·6	0/15	+2·2
1	30	30·8 ± 5·4	6/15	−0·9
1	15	23·4 ± 3·0	3/15	+2·4
1	7·5	13·4 ± 1·8	0/15	+0·4
1	3·75	36·2 ± 6·0	9/15	+10·0
0·5	30	24·0 ± 3·9	3/15	+0·8
0·5	15	21·0 ± 4·5	3/15	+1·4
0·5	7·5	24·0 ± 1·6	0/15	+2·4
0·5	3·75	26·0 ± 5·1	3/15	+2·8
(B) Ehrlich-thioguanine resistant subline				
0	0	10·7 ± 0·9	0/10	+2·97
0	30	14·3 ± 2·3	0/10	+4·76
0	15	14·2 ± 1·9	0/10	+5·7
1	0	8·1 ± 0·8	0/10	+5·3
0·5	0	10·2 ± 1·0	0/10	+4·2
1	30	8·1 ± 1·9	0/10	−3·1
0·5	15	9·4 ± 2·0	0/10	+4·0

* Average deviation from mean.

time markedly beyond that produced by any dose of either drug alone. These combinations also resulted in mice surviving 50 days; almost all of the surviving animals were tumor-free at that time. The thioguanine-resistant subline of the Ehrlich ascites carcinoma showed some cross-resistance to 6-mercaptopurine, but this resistance was not as complete as that to thioguanine (Table 1B). Treatment with combinations of thioguanine and 6-mercaptopurine, which showed potentiation in the parent line, here showed some increased toxicity.

The mechanism of action of neither of these drugs is known. These biological data, however, support implications of other workers that their carcinostatic activity must involve different biochemical reactions.

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Vol. 5 1960

On the mechanism of the convulsivant effect of hydrazides in mice

(*Received* 16 *June* 1960)

METHODS

1. *Manometric estimation of glutamic acid decarboxylase activity of the brain.* Two ml homogenate 1 : 4, 0·067 M phosphate buffer, pH 6·5; 40 mg Na-glutamate in 0·3 ml distilled water, 37 °C, N_2 atmosphere.

2. *Amino acid estimation.* γ-Aminobutyric acid (GABA), glutamic acid, aspartic acid. For each experiment three mice brains were homogenized with 5 ml 70% ethanol. Separation of the amino acids of the alcoholic extracts by high voltage electrophoresis; direct quantitative estimation on the paper with the "Spinco Analytrol" (Beckman).

RESULTS

It is known that after the injection of hydrazines[1, 2] in convulsive doses the GABA content of the brain is decreased.[3] The cause is an inhibition of the glutamic acid decarboxylase. The hydrazides form hydrazones with the coenzyme pyridoxal-5-phosphate.

(1) Pyridoxine which inhibits the convulsions does not prevent the decrease of the GABA-content of the brain. On the contrary, after simultaneous injections of pyridoxine and hydrazides the fall in GABA content was even more pronounced (Table 1), in spite of a normal activity of the glutamic acid decarboxylase (Table 2, thiosemicarbazide). The explanation probably is that pyridoxine catalyses the

TABLE 2. THE EFFECT OF PYRIDOXINE ON THE *in vivo* INHIBITION OF GLUTAMIC ACID DECARBOXYLASE ACTIVITY OF THE BRAIN CAUSED BY HYDRAZIDES

	μl. CO_2 90 min	% Activity
Controls	55	100
Pyridoxine 50–300 mg/kg i.m.	71	129
Thiosemicarbazide 16 mg/kg i.m. (CD100 + 25%)	34	62 (−38)
Thiosemicarbzide 16 mg/kg i.m. + pyridoxine 50 mg/kg	55	100
Isonicotinic hydrazide 200 mg/kg i.m. (CD100)	28	51 (−49)
Isonicotinic hydrazide 200 mg/kg i.m. + pyridoxine 300 mg/kg (simultaneously)	38	69 (−31)
Isonicotinic hydrazide 200 mg/kg i.m. + Pyridoxine 300 mg/kg (30 min before INH)	19	35 (−65)

The mice were sacrificed 60 min after the injection of hydrazides (45 min after injection of INH).

transamination between GABA and α-ketoglutaric acid more strongly than the decarboxylation of glutamic acid.

It is concluded that the seizures are caused not by the actual decrease in brain GABA, but rather by the reduced turnover in the glutamic acid-GABA-α-ketoglutaric acid metabolism.

(2) Seizures caused by isonicotinic acid hydrazide (INH) were not prevented by pyridoxine (Py). Simultaneous administration of pyridoxine reduced the inhibition of decarboxylase by INH; if Py was injected 30 min before INH, it enhanced the inhibition (Table 2). After high doses of Py-5-phosphate the INH-seizures occurred earlier than after INH alone.

TABLE 1. THE EFFECT OF HYDRAZIDES AND PYRIDOXINE ON THE GABA GLUTAMIC ACID AND ASPARTIC ACID CONTENT OF THE BRAIN

	No. of mice	No. of exp.	γ-Aminobutyric acid			Glutamic acid			Aspartic acid
			μmole/g	decrease	% significance	μmole/g	decrease	% significance	μmole/g
Controls	180	60	3·49 ± 0·14			17·5 ± 0·38			4·38 ± 0·11
Thiocarbohydrazide 4 mg/kg i.m.	21	7	2·07 ± 0·19	−40	$t = 3·46$ $p = 0·001$	14·5 ± 1·27	−17	$t = 5·22$ $p = 0·001$	3·62 ± 0·31
Thiosemicarbazide 13 mg/kg i.m.	57	19	3·56 ± 0·21	0		17·7 ± 0·84	0		4·20 ± 0·25
Isonicotinic hydrazide 200 mg/kg i.m.	27	9	1·84 ± 0·22	−47	$t = 4·25$ $p = 0·001$	13·5 ± 1·09	−23	$t = 3·15$ $p = 0·01$	4·66 ± 0·42
Thiocarbohydrazide 4 mg/kg i.m. +pyridoxine 50 mg/kg	21	7	1·32 ± 0·13	−62	$t = 5·3$ $p = 0·001$	14·1 ± 0·96	−19	$t = 2·9$ $p = 0·01$	3·98 ± 0·17
Thiosemicarbazide 13 mg/kg i.m. +pyridoxine 50 mg/kg	24	8	2·50 ± 0·24	−28	$t = 2·5$ $p = 0·02$	13·5 ± 1·06	−23	$t = 2·4$ $p = 0·01$	3·51 ± 0·42
Isonicotinic hydrazide 200 mg/kg i.m. +pyridoxine 600 mg/kg	12	4	1·64 ± 0·23	−53	$t = 3·36$ $p = 0·01$	16·7 ± 1·32	− 5		3·77 ± 0·32

Pyridoxine and convulsive doses of the hydrazides simultaneously were injected i.m. to groups of three mice; 60 min later the animals were sacrificed.

It is concluded that Py and Py-5-phosphate are not merely biochemical antagonists of INH, but can potentiate the action of INH, perhaps by facilitating the penetration of INH into the brain across the blood–brain barrier.

Nicotinamide (400–500 mg/kg) abolished the seizures caused by INH (200–250 mg/kg) in 50 per cent of the experimental animals.

TABLE 3. THE EFFECT OF RESERPINE ON THE GABA-CONTENT OF THE BRAIN AND THE THRESHOLD OF ELECTROSEIZURES IN MICE

	Numbers of mice (n)	Numbers of exp.	γ-Aminobutyric acid			Glutamic acid (μmole/g)	Aspartic acid (μmole/g)	threshold for electro-seizures (100 Hz, 5 msec, 5 sec)
			μmole/g	% de-crease	signifi-ance			
Controls	$n = 180$	60	3·49 ± 0·14			17·5 ± 0·38	4·38 ± 0·11	5 mA ($n = 10$)
Reserpine 5 mg/kg s.c. 3 hr before injection	$n = 30$	10	2·42 ± 0·51	−30	$t = 2·67$ $p = 0·01$	17·7 ± 1·71	3·96 ± 0·38	1·0–2·5 mA ($n = 10$)

(3) The *tonic* component of hydrazide induced seizures was abolished when the dopamine content of the brain was raised by the injection of iproniazid (100 mg/kg i.m.) and L-DOPA (100 mg/kg i.p., 2 hr before). This may explain why low doses of pyridoxine (18 mg/kg) antagonized the convulsive action of *iso*propylhydrazine (400 mg/kg), although they had only a weak effect on the inhibition of glutamic acid decarboxylation: *iso*propylhydrazine is a potent inhibitor of monoamine oxydase.

(4) Doses of reserpine which decreased the threshold for electroshock, diminished the content of GABA in the brain, but not that of glutamic and aspartic acid (Table 3).

(The results of our experiments will be published *in extenso* in *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.*)

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Papers to be published in future issues

A. Winkler, J. Škoda, V. Ujházy, V. Černý, L. Šándor and F. Šorn: Excretion of 6-azauracil ribroside in tumerous diseases.

C. E. Purdom: Mut a genic effects of nitrogen mustard deviatives of azo-benzene compounds in *Drosphila melogaster*.

Fabian J. Lionetti, Norman C. Lelles and Nicholas C. Avery: Inhibition of dehydrogenase of human leukocytes by hexetidine.

Anders Sundwall: Plasma concentration curves of N-methylpyridinium-2-aldodime methane sulphonate (P2S) after intravenous intramuscular and oral administration in man.

P. Alexander and Z. B. Mikulski: Differences in the response of leukaemia cells in tissue culture to nitrogen mustard and to dimethyl myleran.

A. W. Dahl, T. L. Fletcher, H. H. Skinner, Jr. and K. Merendino: Fibrosing properties, in the rat, of compounds related to dicetyl-(dl-*n*-hexadecyl)-phosphate—I.

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Alan C. Sartorelli and Barbara A. Booth: Utilization of preformed purines by sarcoma 180 or hepatome 134 cells treated with 6-chloropurine or a combination of 6-chloropurine and azaserine.

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Kenneth E. Moore and Theodore M. Brody: The effect of triethyltin on mitochondrial swelling.

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ERRATUM

R. G. Wiegand and J. D. Taylor: Kinetics of plasma drug levels after sustained release dosage. **3**, 256–263

Page 258, Formula (8) should read:

$$b = \frac{a_o f_r k_a k_r}{(k_a - k_r)(k_e + k_m - k_r)}(e^{-k_r t} - e^{-(k_e + k_m)t}) + \frac{a_o f_r k_a - \frac{a_o f_r k_a k_r}{(k_a - k_e)}}{(k_e + k_m - k_a)}(e^{-k_a t} - e^{-(k_e + k_m)t})$$

Biochemical Pharmacology, 1960, Vol. 5, pp. 173–186. Pergamon Press Ltd., Printed in Great Britain.

ARYLESTERASES IN BLOOD: EFFECT OF CALCIUM AND INHIBITORS

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(*Received* 11 *April* 1960)

Abstract—(1) The normal functioning of human serum arylesterase requires the presence of calcium. An enzyme in human plasma fraction IV-1 is probably identical with serum arylesterase. The activity of arylesterase is accelerated by calcium, inhibited by various chelating agents, metal ions, SH-reagents, and competitively and reversibly inhibited by the ionized form of 1-phenyl-2-hydrazinopropane. The D-isomer of this compound is more effective than the L-isomer. In contrast to results with arylesterase, the racaemic form of this agent accelerated the hydrolysis of phenyl acetate by human plasma cholinesterase. The best inhibitors of arylesterase were the rare earths, some of them active in the order of 10^{-8} M. Under the experimental conditions used calcium chloride protected the enzyme partially or fully against the inhibition and against loss of activity during dialysis.

(2) Swine serum contains an arylesterase with properties similar to those of human serum.

(3) The human red blood cell and serum arylesterase are not identical.

ALDRIDGE[1, 2] described in 1951–1953 the existence of an A esterase in mammalian blood sera. This enzyme hydrolyzes *p*-nitrophenyl acetate and diethyl *p*-nitrophenyl phosphate. It is probably identical with the aromatic esterase (Ae)* of Mounter and Whittaker[3] which is mainly responsible for the hydrolysis of PA in human plasma. The aryl (aromatic) esterase and the cholinesterase, the other enzyme in human plasma which can use the same substrate, were separated electrophoretically by Augustinsson[4] and by Marton and Kalow.[5] The dependence on calcium of the Ae as well as its inhibition by various agents was described by Erdös *et al.*[6] Some of the experiments given here in detail were summarized in the same article.

MATERIALS AND METHODS

The majority of the experiments were carried out in a Cary no. 11 recording u.v. spectrophotometer using a modification of Zeller's[7] method. The instrument was equipped with a 0–2·4 and with a 0–0·1 slide wire assembly, which increased the extent of registration on the chart paper twenty-four-fold. Four-milliliter absorption cells were used with a 10-mm light path. The temperature was kept constant at 27 °C and the concentration of PA was 1×10^{-3} M. Generally, activators or the inhibitors were pre-incubated for 5 min at 27 °C with the enzyme. The recording of the increase

* Abbreviations: Ae = aryl (aromatic) esterase; PA = phenyl acetate; tris = tris-hydroxymethyl-aminomethane; Å = Ångström; EDTA, Ca-EDTA, Mg-EDTA, Zn-EDTA = sequestrene: disodium ethylenediamine tetraacetic acid and its Ca, Mg, and Zn complexes; JB516 = dl-1-phenyl-2-hydrazinopropane HCl; DFP = diisopropylfluorophosphate.

in optical density started 30 sec after mixing the substrate and the enzyme. A 0·025 M tris buffer of pH 7·3 was employed. (The activity of the serum was markedly less in a phosphate buffer.) The hydrolysis was followed for 5–10 min; the results were calculated from the initial steady rates. Fig. 1 shows the changes in optical density during the hydrolysis of 1×10^{-3} M PA by the Ae in fraction IV-1 of human plasma. The u.v. range of the spectrophotometer was scanned at a rate of 10 Å/sec. The initial (PA) and the final (phenol) absorption spectra are marked A and B, respectively. In most of the routine studies a wavelength of 2800 Å was used.

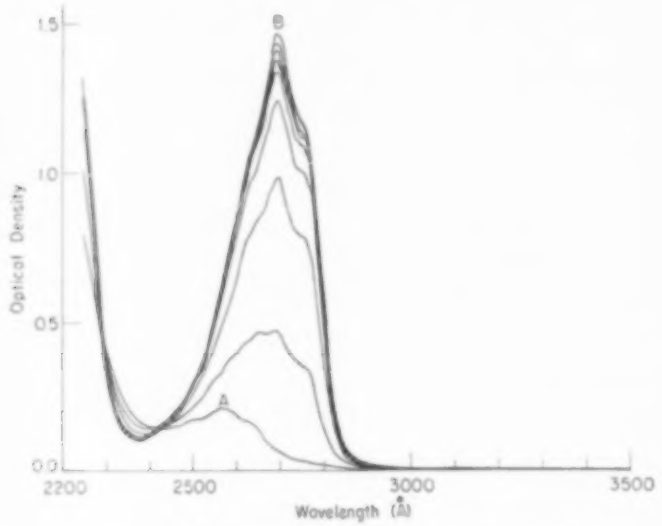

Fig. 1. Continuous scanning of the hydrolysis of phenyl acetate by arylesterase. A is the initial, B the final absorption spectrum.

Unlike the spectrophotometric technique, which measured the rate of hydrolysis of PA by determining the amount of phenol liberated, control studies assayed the amount of acid released by the enzymes. Here a Warburg manometric apparatus or a Radiometer automatic recording titrator was employed at 27 °C. With the conventional manometric method a $NaHCO_3$ solution of 0·031 M and a substrate concentration of 4×10^{-3} M were used. The time of pre-incubation was 20 min.

In the titrator the enzyme was assayed in 10 ml of NaCl solution, 0·025 M, by maintaining a constant pH (7·4), with 0·01 N, or in the red blood cell experiments; with 0·02 N NaOH. The concentration of substrate was 1×10^{-3} M.

In the earlier part of the investigation, human plasma, later pooled normal human serum, was used with similar results. During the experiments with the spectrophotometer a stock solution of serum in tris (1 : 100 v/v) was kept in an ice bath; subsequent dilutions were made 5–10 min before starting the reaction. The final dilution of serum was 1 : 2000 v/v with this technique; it was 1 : 1000 v/v with the manometric method. At this dilution only a negligible amount of PA was hydrolyzed by the cholinesterase present in the sera of healthy donors. The results with some sera were discarded because the preparation of the enzyme became unstable.

Human plasma fractions were obtained from the Protein Foundation, Inc. and from Cutter Laboratories. Swine serum was pooled from ten pigs (Colorado Serum Company). Swine plasma fractions were purchased from Pentex Corporation. The activity of plasma fractions was determined in a manner similar to that of human serum. The source of purified human plasma cholinesterase was fraction IV-6-1-2 (Cutter Laboratories), which is equivalent to Cohn fraction IV-6-3. Human red blood cells were washed three times in physiological saline and haemolyzed in distilled water. The final dilution of packed red blood cells was 1 : 60 v/v.

Dialysis experiments were carried out in an Oxford multiple dialyzer with Visking seamless cellulose tubing. The tubes contained 5 ml of enzyme solution which was dialyzed against 4 l. of tris of pH 7·4 at 2–4 °C. When indicated in the tables, EDTA or Ca-EDTA was added to this solution of enzyme. The activity of dialyzed Ae was compared with a sample stored in the refrigerator. During the 40-hr dialysis run, three changes of buffer were used. The dilutions of enzymes in tris in the dialysis tubes or during storage were: human serum, 1 : 10 v/v; fraction IV-1, 5 mg/ml; swine serum, 1 : 10 v/v. The final dilutions in the spectrophotometer were: human serum, 1 : 2000 v/v; fraction IV-1, 0·05 mg/ml; swine serum, 1 : 400 v/v.

When the enzyme was dialyzed against calcium, the same concentration of calcium chloride was used inside and outside the dialysis bags.

RESULTS

Human serum Ae

1. *Effect of chelating agents and calcium.* Our interest in the role of calcium in Ae stems from the observation that EDTA, added to prevent the coagulation of blood samples, inhibited Ae without affecting the activity of cholinesterase. EDTA at a concentration of $2{\cdot}5 \times 10^{-5}$ M inhibited the enzyme completely in the spectrophotometer. On the other hand, EDTA in concentrations less than 10^{-5} M enhanced the enzymic activity. We attribute this activation to the binding of an inhibitor present in our system. The character of the inhibition was further explored with the help of various sequestering agents. It is known that, to an extent largely determined by their respective stability constants (K),* cations can replace each other in the EDTA complex. Thus, for example, Ca^{2+} (log $K = 10{\cdot}6$) can replace Mg^{2+} (log $K = 8{\cdot}7$). Accordingly, in our experiments Mg-EDTA inhibited to approximately the same degree as EDTA, possibly through exchanging Mg^{2+} for Ca^{2+} in the system. Conversely, Zn-EDTA (log $K = 16{\cdot}6$) was an ineffective inhibitor. Calcium-EDTA also did not inhibit, but accelerated about 50 per cent in the 10^{-4}–10^{-5} M range. Chel DP (ethylenediamine di (O-hydroxyphenyl acetic acid)) which has little tendency to co-ordinate with alkaline earth ions (log K for $Ca^{2+} = 1{\cdot}6$) was similar to Ca-EDTA. Chel DM (hydroxyethyl ethylenediamine triacetic acid) is, on the other hand, a good inhibitor. Its log K for Ca^{2+} is 8·0, and it inhibits Ae 90 per cent at a 5×10^{-5} M concentration. Sodium citrate (see Fig. 2) also inhibited at a somewhat higher concentration ($I_{50} = 7 \times 10^{-4}$ M).

The inhibition of the enzyme by EDTA could be reversed by the addition of gelatin, ashed gelatin, or calcium to the system. These agents also accelerated the

* The stability constants of the chelating agents do not show the actual values under the present experimental conditions, but were taken from the literature to compare them against each other under identical conditions.

hydrolysis of PA. The various sera or plasma samples were accelerated by Ca^{2+} to varying extents. Experiments with nineteen different enzyme preparations gave a mean value of 83 per cent (s.e. = ± 9) acceleration by 1×10^{-4} M $CaCl_2$.

When a 1×10^{-4} M $CaCl_2$ solution was rapidly added into the absorption cell of the spectrophotometer, which already contained 5×10^{-5} M EDTA, enzyme and substrate, the inhibition gradually decreased and up to 70 per cent of the normal activity was restored. When $MgCl_2$ was used instead of $CaCl_2$, the reactivation was negligible.

2. *Effect of dialysis.* The importance of calcium was also seen in dialysis studies. Table 1 summarizes the results with human serum Ae. On the average, the activity of the enzyme decreased 65 per cent during the dialysis, but it was partially restored by adding calcium to the solution of enzyme. When the dialysis tube contained EDTA at the beginning of the dialysis, 94 per cent of the activity disappeared and calcium did not reactivate the enzyme. This indicates that the removal of calcium from the protein led to irreversible changes. Storing the enzyme with EDTA also abolished the activity. When the enzyme contained Ca-EDTA, the activity decreased during dialysis somewhat less than the control and increased during storage. Finally, the enzyme remained stable when dialyzed against 1×10^{-4} M $CaCl_2$.

TABLE 1. EFFECT OF DIALYSIS ON HUMAN SERUM ARYLESTERASE

	Activity in the presence of:					
	—		EDTA		Ca-EDTA	
Enzyme*		+ 1×10^{-4} M $CaCl_2$		+ 1×10^{-4} M $CaCl_2$		+ 1×10^{-4} M $CaCl_2$
I (stored)	100	177	1	16	153	204
II (dialyzed)	35	73	6	6	44	74
III (dialyzed)†	98	157	—	—	—	—

* Human serum was stored (I) or dialyzed (II, III) against tris for 17 hr. The concentration of EDTA or Ca-EDTA in the solution of the enzyme at the start of the experiments was 1×10^{-2} M, the final dilution in the non-dialyzed sample 5×10^{-5} M. The activity of the stored enzyme = 100.

† Dialyzed against 1×10^{-4} M $CaCl_2$ in tris.

3. *Effect of inhibitors.* The results with calcium prompted us to investigate the effect of metal ions and other agents on this enzyme which has been reported to be insensitive to a number of commonly used inhibitors.[4] Figs. 2 and 3 show the inhibition of serum Ae. Salts of the rare earth series and yttrium had the lowest I_{50} values. These trivalent cations inhibited the enzyme under our experimental conditions in the 10^{-7}–10^{-8} M range. Heavy metals like $CdSO_4$, $HgCl_2$, $AgNO_3$, etc., were also very active. It is of interest that even in tris buffer, Cu^{2+} was a good inhibitor. The sensitivity of the enzyme to the metal ions varied from one serum sample to another. Figs. 2 and 3 represent the mean values of the results. The I_{50} values in M were as follows: $GdCl_3$, $CeCl_3$, 4×10^{-8}; $LaCl_3$, 6×10^{-8}; $SmCl_3$, $Y(NO_3)_3$, 2×10^{-7}; $CdSO_4$, 3×10^{-7}; $HgCl_2$, $AgNO_3$, 7×10^{-7}; $PbCl_2$, 2×10^{-6}; $ZnSO_4$, 1×10^{-6}; $NiSO_4$, $CoSO_4$, 3×10^{-6}; $CuCl_2$, 8×10^{-6}; $MnSO_4$, 1×10^{-5}; $BaCl_2$, 6×10^{-5}; $SrCl_2$, 9×10^{-5}; $MgCl_2$, 4×10^{-4}.

The inhibition by the less basic metals, with some exceptions, follows their affinity toward sulfide, a circumstance which indicates that the inhibition might be due to attachment to free SH-groups of the protein. In addition to metals, the enzyme was also inhibited by the SH-reagent, *p*-chloromercuriphenylsulfonic acid, $I_{50} = 3 \times 10^{-6}$, and by the amine oxidase inhibitor,[6, 9] JB516, $I_{50} = 2 \times 10^{-4}$.

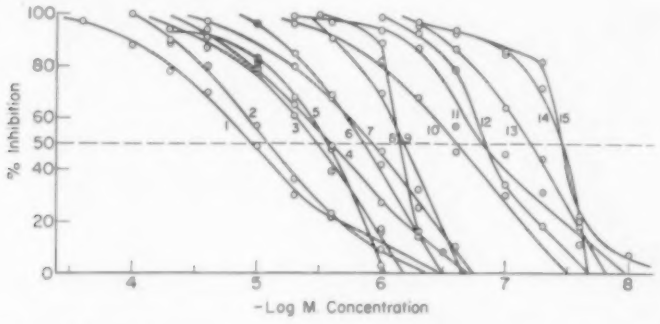

FIG. 2. Inhibition of the arylesterase of human serum. (1) $MnSO_4$; (2) $CuCl_2$; (3) $CoSO_4$; (4) *p*-chloromercuriphenylsulfonic acid; (5) $NiSO_4$; (6) $ZnSO_4$; (7) $PbCl_2$; (8) $AgNO_3$; (9) $HgCl_2$; (10) $CdSO_4$; (11) $Y(NO_3)_3$; (12) $SmCl_3$; (13) $LaCl_3$; (14) $GdCl_3$; (15) $CeCl_3$.

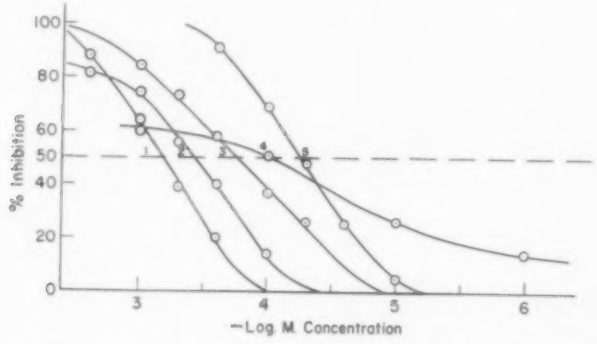

FIG. 3. Inhibition of the arylesterase of human serum: (1) sodium citrate; (2) $MgCl_2$; (3) JB516; (4) $SrCl_2$; (5) $BaCl_2$.

Because some enzyme preparations became unstable after a longer period, only 5 min were allowed for pre-incubation in the spectrophotometric studies. Experiments using longer periods of pre-incubation, with basically similar results, were done with the manometric method or with the more stable purified enzyme.

The inhibition by $AgNO_3$ was determined only with the manometric technique. With this method $CuCl_2$, $NiSO_4$, $HgCl_2$ and EDTA inhibited to about the same extent as in the spectrophotometric experiments.

Because $HgCl_2$ and *p*-chloromercuriphenylsulfonic acid are among the agents which are considered to react with the SH-groups of enzymes, an attempt was made to reverse inhibition of human Ae by adding glutathione. The experiments, resembling those of Aldrige[1] with rabbit plasma, show that glutathione partially restored the activity of Ae.

Table 2. Reactivation of human serum arylesterase inhibited by SH-reagents*

Inhibitor	Rate†
1×10^{-6} M $HgCl_2$	27
1×10^{-6} M $HgCl_2 + 1 \times 10^{-3}$ M glutathione	72
1×10^{-5} M CMPS‡	9
1×10^{-5} M CMPS $+ 1 \times 10^{-3}$ M glutathione	55

* Experiments were done in a Warburg manometric apparatus; 15 min after the inhibitor had been added to the enzyme, glutathione was dropped from a side arm into the main compartment of the vessels; then 15 min later the substrate was dropped from a second side arm.
† Uninhibited rate = 100.
‡ *p*-Chloromercuriphenylsulfonic acid.

Ae in fractions of human plasma

The Ae content of various fractions of human plasma was investigated in the hope of finding a source of more stable enzyme than that represented by diluted serum. Fraction IV-1 contained an enzyme with properties very similar to those of human serum Ae. Experiments with accelerators and inhibitors yielded qualitatively the same results. The highest sensitivity to activator or inhibitors was encountered in human serum, but the stability of the enzyme in fraction IV-1 exceeded that of other sources. EDTA inhibited the enzyme in fraction IV-1 completely at 5×10^{-5} M concentration. $CaCl_2$ reactivated the enzyme inhibited by EDTA. To a solution of Ae in fraction IV-1, 5×10^{-5} EDTA was added and 5 min later 1×10^{-4} M $CaCl_2$. After another 5 min the substrate was mixed with the enzyme. In contrast to the control, calcium completely abolished the inhibition. Furthermore, dialysis studies showed that the dependence of the enzyme on calcium had not changed during the purification.

Table 3. Effect of dialysis on arylesterase in human plasma fraction IV-1

	Activity in the presence of:					
	—		EDTA		Ca-EDTA	
Enzyme*		2×10^{-4} M $CaCl_2$		2×10^{-4} M $CaCl_2$		2×10^{-4} M $CaCl_2$
I (stored)						
17 hr	100	144	0	26	133	165
40 hr	100	142	0	15	137	170
II (dialyzed)						
17 hr	57	73	7	7	67	75
40 hr	30	36	5	5	26	39
III (dialyzed)†						
17 hr	97	140	—	—	—	—
40 hr	91	136	—	—	—	—

* The arylesterase was stored (I) or dialyzed (II, III) against tris for 17 and 40 hr, respectively. The concentration of EDTA or Ca-EDTA in the solution of enzyme at the start of the experiments was 1×10^{-2} M, the final dilution in the non-dialyzed sample 1×10^{-4} M. The activity of the stored enzyme = 100.
† Dialyzed against 3×10^{-4} M $CaCl_2$. The activity of the stored enzyme which contained $CaCl_2$ = 100.

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Table 3 shows that the Ae activity of fraction IV-1 decreases during dialysis; however, it can be partially reactivated with calcium. When the enzyme was stored in the presence of EDTA, the activity disappeared, but it was restored in part by calcium. The removal of calcium with EDTA during dialysis led to irreversible changes. Like that of serum Ae, the activity of the enzyme increased during storage in the presence of Ca-EDTA and behaved during dialysis similarly to the untreated control. The activity of fraction IV-1 remained constant when dialyzed against 3×10^{-4} M $CaCl_2$.

A hydrolytic enzyme for PA was also found in fractions IV-4 and IV-6-3. Others have reported[10] that this enzyme is identical with plasma cholinesterase. In spectrophotometric studies, traces of Ae activity were also seen in the clear supernatant of fractions III and III-0. When the soluble portions of the fractions were not separated in the centrifuge, but were tested as a homogenate in the automatic titrator, fractions III and III-0 showed more activity than previously. But even then the enzyme content of fraction III was less than 15 per cent of that of IV-1.

Inhibition of purified Ae

The inhibition of the enzyme in fraction IV-1 was comparable to that of human serum. It was sensitive to cations of rare earths and other metals. JB516 also inhibited the enzyme. To decide whether the charged or the uncharged form of this amine oxidase inhibitor ($pK_a = 7{\cdot}31$)[11] is responsible for the inhibition, the activity of the enzyme was measured in the presence and absence of the inhibitor at various pH values, within the range of the tris buffer. The degree of inhibition was calculated from the uninhibited control rates at each pH value and it seemed to parallel roughly the percentage ionization[12] of this compound.

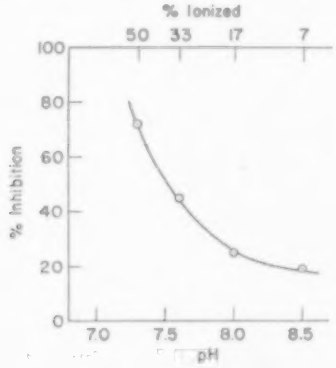

FIG. 4. Dependence of the inhibition of arylesterase in fraction IV-1 by JB516 (1×10^{-3}) on the pH and on the ionization of this inhibitor.

In contrast to this, $LaCl_3$ inhibited at pH 7·3 and 8·5 to about the same extent. The accelerative effect of JB516 on plasma cholinesterase also depends upon the cationic form[13].

The importance of the hydrazine group in the side chain of this compound is shown by the fact that the arylalkylamine congeners of JB516, D- or L-amphetamine (1-phenyl-2-aminopropane) inhibited less than 10 per cent at 10^{-3} M concentration. In

the experiments described above the racemic form of the inhibitor was used. When D- and L-isomers were tested individually, a small but significant difference was found. D-1-phenyl-2-hydrazinopropane was a better inhibitor than the L analog. Fig. 5 compares the D- and L-forms; the I_{50} values were 4×10^{-4} and 2×10^{-4} M, respectively. The I_{50} for the longer homolog of the series D, L-1-phenyl-3-hydrazinobutane is also 2×10^{-4} M. The activity of racemic JB516 falls between those of its D- and L-derivatives.

In the present studies the Ae was inhibited reversibly by JB516. When experiments were carried out with the Ae in fraction IV-1, analogously to those described above above with calcium and EDTA, 1×10^{-3} M calcium fully reversed the inhibition of the enzyme by 1×10^{-3} M JB516.

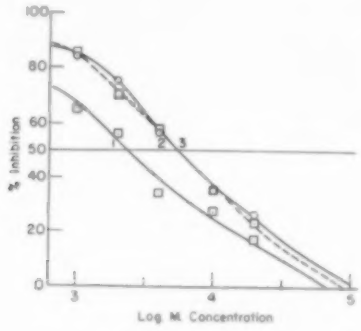

FIG. 5. Inhibition of arylesterase in fraction IV-1 by L-1-phenyl-2-hydrazinopropane (1); D, L-1-phenyl-3-hydrazinobutane (2); and by D-1-phenyl-2-hydrazinopropane(3).

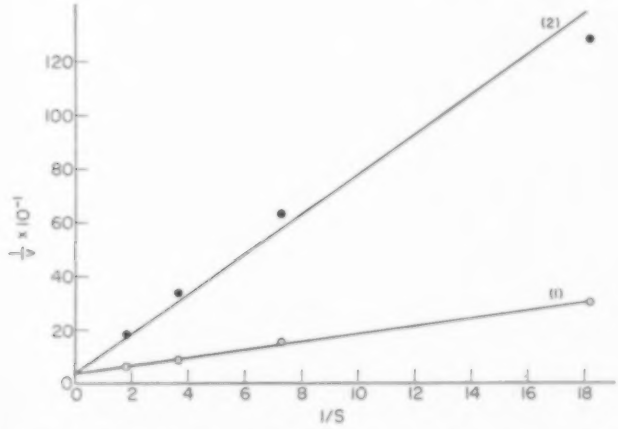

FIG. 6. Competitive inhibition of the hydrolysis of phenyl acetate by arylesterase in fraction IV-1 (1). Inhibitor: JB516 (2). Concentration of substrate and rates were expressed in optical density units.

The type of inhibition was further investigated by the method of Lineweaver-Burk. Figure 6 shows the results. The K_m of Ae from this curve is 8×10^{-4} M and JB516 seems to be a competitive inhibitor of the enzyme.

The inhibition of Ae by $HgCl_2$ or $LaCl_3$ could be clocked by calcium. In Fig. 7 the

reciprocal values of the M concentrations of calcium, 1/Ca, were plotted, according to Nishi,[14] against the reciprocal values of velocity, $1/V$, in the presence and in the absence of the inhibitors, $LaCl_3$ and $HgCl_2$. The inhibition decreased with increasing calcium concentration, but the rate of hydrolysis of the uninhibited, activated enzyme was not reached even in the presence of 3×10^{-3} M $CaCl_2$. The intercept, V_{max}, was different for the inhibited and uninhibited reaction.

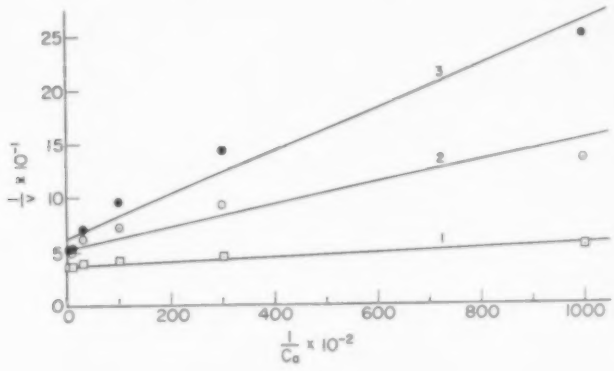

FIG. 7. Effect of increasing the concentration of $CaCl_2$ (1) on the inhibition of arylesterase in fraction IV-1 by 1×10^{-6} M $HgCl_2$ (2) and $LaCl_3$ (3). Uninhibited reaction (1). Regression lines 2 and 3 were calculated according to Finney[15]. Ordinate: Reciprocals of rate in optical density units. Abscissa: Reciprocals of M concentration of $CaCl_2$.

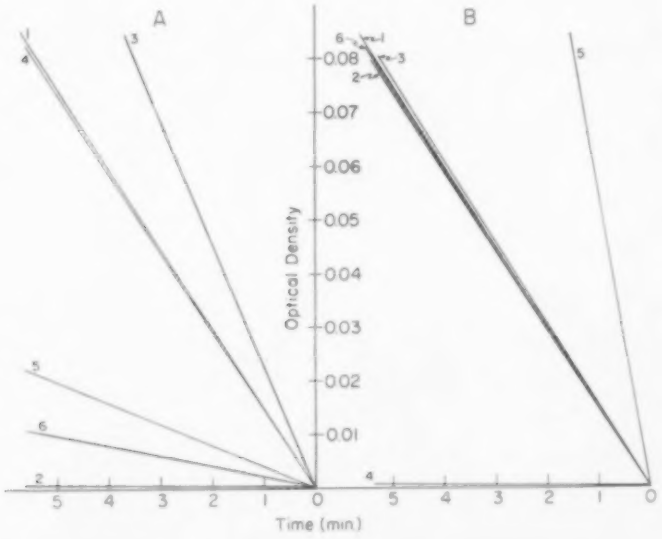

FIG. 8. Inhibition and acceleration of the hydrolysis of phenyl acetate by arylesterase in fraction IV-1 (A) and cholinesterase in fraction IV-6-3 (B) of human plasma: (1) control; (2) EDTA 5×10^{-5} M; (3) $CaCl_2$ 1×10^{-4} M; (4) eserine $2{\cdot}5 \times 10^{-6}$; (5) JB516 1×10^{-3}; (6) $LaCl_3$ 1×10^{-6}.

In a few control studies with various inhibitors, the time of pre-incubation was increased from 5 to 60 min without a significant increase in the percentage inhibition.

Ae belongs to the SH-enzymes which are sensitive to inhibition by urea. Calcium protects the enzyme against this type of inhibition also: the problem will be dealt with in detail elsewhere.

The differences between the Ae in fraction IV-1 and the cholinesterase in IV-6-3 are summarized in Fig. 8. The enzymes were assayed with the same u.v. spectrophotometric technique. Fraction IV-6-3 was diluted 1 : 2000 v/v from a 35 mg/ml stock solution; fraction IV-1, 1 : 100 v/v from a 5 mg/ml stock solution. In this figure the increase in optical density is plotted against time in minutes. Part A shows that the Ae was inhibited by EDTA, JB516 and $LaCl_3$, activated by $CaCl_2$ and unaffected by eserine. The activity of fraction IV-6-3 with PA substrate was accelerated by JB516, unchanged by $CaCl_2$ (1×10^{-4} M), EDTA or $LaCl_3$ and, as expected, inhibited by eserine.

Ae in swine plasma

Like human serum, swine plasma contains only two enzymes which hydrolyze PA: a cholinesterase and an Ae.[16] The Ae was also very sensitive toward the various inhibitors tried here. At 1 : 400 v/v dilution, the swine serum was inhibited 95 per cent by 1×10^{-4} M EDTA, 98 per cent by 1×10^{-4} M Mg-EDTA, 62 per cent by 1×10^{-3} M JB516; Ca-EDTA did not inhibit. Other inhibitors of human Ae were also active. At 1 : 1000 v/v dilution at a wavelength of 2700 Å the following inhibition was observed: $2{\cdot}5 \times 10^{-7}$ M $GdCl_3$ and $LaCl_3$, 68 and 76 per cent, respectively; 5×10^{-5} M *p*-chloromercuriphenylsulfonic acid, 50 per cent.

Table 4. Effect of dialysis on arylesterase in swine serum

	Activity in the presence of:					
	—		EDTA		Ca-EDTA	
Enzyme*		+ 1×10^{-3} M $CaCl_2$		+ 1×10^{-3} M $CaCl_2$		+ 1×10^{-3} M $CaCl_2$
I (stored)						
17 hr	100	97	4	81	108	110
40 hr	100	94	3	58	109	102
II (dialyzed)						
17 hr	86	90	46	49	95	90
40 hr	83	78	30	29	81	80

* Swine serum was stored (I) or dialyzed (II) against tris for 17 and 40 hr, respectively. The concentration of EDTA or Ca-EDTA in the solution of enzyme at the start of the experiments was 4×10^{-3} M, the final dilution in the non-dialyzed samples 1×10^{-4} M. The activity of the stored enzyme = 100.

In variance to that with human Ae, the observed rate of hydrolysis of PA in swine plasma did not increase in the presence of added calcium. Therefore, the role of calcium

was also investigated in dialysis studies. Even during the 40-hr run, the swine Ae level decreased but little. The presence of Ca-EDTA had no significant effect; on the other hand, 97 per cent of the activity disappeared during the storage with EDTA. Depending on the length of storage, the enzyme can be reactivated in part by adding calcium to the system. The dialyzed sample which contained EDTA at the beginning of the experiment lost 70 per cent of its activity in 40 hr. Here calcium did not reactivate. Thus, the experiments suggest that the inhibition of the enzyme is reversible in the presence of bound calcium. The removal of calcium from the medium leads to irreversible changes.

The distribution of the enzyme in the fractions of swine serum was less well defined than in human. The activity of the various samples was assayed with the manometric technique. EDTA (1×10^{-3} M) and eserine (1×10^{-5} M) were used as inhibitors. Ae was found in fractions I and III and cholinesterase in fractions II and IV.

Red blood cell Ae

The existence of three enzymes in human red blood cells, all capable of hydrolyzing PA, was described by Mounter and Whittaker.[3] The first enzyme, a cholinesterase, is inhibited by eserine, the second one, an aliesterase, by DFP. Lacking a specific inhibitor, these authors assumed that the third enzyme would be identical with plasma Ae. We have studied this enzyme in the presence of eserine, 1×10^{-5} M, in the automatic recording titrator. DFP achieved only 5 per cent additional inhibition, a finding which indicates that at the 1×10^{-3} M substrate level, the aliesterase did not play an important role in the overall hydrolysis rate. After 5 min of pre-incubation the activity of the third enzyme was remarkably resistant to inhibitors of serum Ae. Both JB516 and EDTA were ineffective at 1×10^{-3} M concentration; $CaCl_2$ did not activate the enzyme. 1×10^{-3} M *p*-chloromercuriphenylsulfonic acid inhibited three samples less than 25 per cent but the Ae in the red blood cells of the fourth individual was inhibited 90 per cent. Sodium fluoride, 1×10^{-2} M, inhibited only 20 per cent the hydrolysis of PA. As with phosphatases, $MgCl_2$ in the same concentration accelerated the enzyme by 31 per cent; 1×10^{-3} M or lower concentrations of $LaCl_3$, $GdCl_3$ and $CeCl_3$ instead of inhibiting, increased the acid release. As the liberation of acid in the titrator also increased in the absence of substrate, it was neither due to an enhancement of Ae activity nor to the rare earth alone,[17] but probably to the catalytic effect of rare earth ions. Trapmann[18] indicated that rare earth cations catalyze the hydrolysis of phosphoric acid esters, carboxylic acid esters, etc. The release of acid from haemolyzed red blood cells could reflect a similar catalytic activity.

The experiment suggests that Ae in serum differs from the Ae in red blood cells.

DISCUSSION

Human serum contains at least two enzymes which hydrolyze PA. One is a cholinesterase, the other one an aryl (aromatic) esterase (Ae). Our previous communication[6] has already indicated that calcium is indispensable for the functioning of the Ae. Independently, and a short time later, Marton and Kalow[5] came to similar conclusions. These authors also suggested that in addition to Ca^{2+}, other bivalent metal ions like Cu^{2+}, and Mn^{2+} might be of some importance. The present experiments do not seem to support this theory.

In the experiments described here, the role of calcium was explored with dialysis

studies and other procedures and by using various sequestering agents. During dialysis, the human enzyme remained stable only when it was dialyzed against calcium. Furthermore, EDTA and Mg-EDTA inhibited the Ae while Ca-EDTA was inactive. Thus, the inhibition by Mg-EDTA could mainly be due to the binding of a cation with an affinity to EDTA equal to or less than that of calcium, and higher than that of magnesium. Except for Ca^{2+}, only Sr^{2+} would approach this requirement, but $SrCl_2$ inhibited only. The experiments with other sequestering agents also point to calcium.

Recently, Bernsohn *et al.*[19] confirmed an earlier observation[20] that calcium increased the hydrolysis rate of PA in human serum. Our experiments contradict their failure to observe an activity loss on dialysis.

The best inhibitors of Ae are rare earth cations and yttrium, all of them active at a very low concentration. This effect is probably one of the strongest biological activities known for rare earths.[21] The inhibition caused by $LaCl_3$—and also by some other agents—is antagonized by calcium. It is of interest that calcium and rare earths have the opposite effect on the coagulation of blood[18] also.

The Ae is inhibited by reagents which are known to combine with the SH- groups of proteins. This inhibition is reversed in part by glutathione, as first shown in rabbit serum by Aldridge[1, 2] who also has noticed the inhibition of this enzyme by heavy metals and magnesium. Later, Underhay[22] and Hobbiger[23] mentioned the sensitivity of human Ae to SH-reagents and metals.

A current publication of Marton and Kalow[24] describes several inhibitors of Ae. The most active agents, like chlorpromazine and naphazoline, inhibited in the 10^{-4} M concentration. Chlorpromazine is also a potent inhibitor of cholinesterases.[25] In contrast to JB516, these inhibitors are not antagonized by calcium.

JB516 is also a useful agent for distinguishing the esterases in fractions IV-6-3 and IV-1. While the observed cholinesterase activity is enhanced by the ionized form of this agent, the Ae in fraction IV-1 is inhibited selectively. Some aspects of this acceleration were discussed briefly previously by ERDÖS *et al.*[26]; the details of this effect will be published elsewhere. It has already been shown by one of us that JB516 inhibits the "true" cholinesterase in snake venom.[27]

The Ae activity of human plasma was found mainly in Cohn fraction IV-1. This was briefly mentioned in an earlier communication by Erdös and Debay.[28] The inhibition and acceleration of the enzyme is very similar to that of human serum Ae. The behavior of this enzyme during dialysis is also comparable to that of serum Ae, but it loses activity somewhat slower than does the serum enzyme.

Augustinsson and Heimbürger[29] described the existence of an enzyme in fraction IV-1 capable of hydrolyzing an organophosphorus inhibitor, tabun. This enzyme is activated by Sr^{2+} and Ba^{2+} and unaffected by Ca^{2+}, La^{3+} or dialysis.[30] Augustinsson and Heimbürger[29] have already indicated that the same fraction can also hydrolyze *p*-nitrophenyl acetate.

After finishing the experimental part of the work, we noticed a recent publication mentioning the existence of Ae in fraction IV-1.[31]

Swine serum contains an aryl and a cholinesterase.[16] The Ae was inhibited by the inhibitors of human Ae. Ca^{2+} did not increase the observed hydrolysis rate, but it reactivated the enzyme which was inhibited by EDTA even after prolonged storage.

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This effect was much more pronounced with swine enzyme than with Ae from human sources. The activity of Ae decreased but slightly after 40 hr of dialysis, a finding which indicates that the calcium is more firmly bound to the swine Ae than to the human, but it could be removed and the enzyme irreversibly inhibited by dialyzing the Ae with EDTA.

A recent report[16] indicated that the Ae in swine serum is a gene-controlled enzyme and is to be regarded as one of the first examples of a direct relation between genes and enzymes in higher animals. Naturally, the authors of the publication could only assay the active form of Ae. Our data suggest that Augustinsson and Olson's[16] results could just as well involve genetic factors having to do with the metal binding as with the total synthesis of the enzyme.

The Ae in red blood cells was remarkably resistant to most of the inhibitors of Ae. It was sensitive to *p*-chloromercuriphenylsulfonic acid, but less than to *p*-chloromercuribenzoic acid.[22] While Mounter and Whittaker[3] assumed the identity of the Ae in plasma and red blood cells, Underhay[22] already indicated some differences between the two enzymes. This observation was based on the different rate of hydrolysis of various substrates by the two Ae. Our experiments with selective inhibitors show that the two enzymes are probably not identical.

Acknowledgements—The writers are thankful for the advice and interest of Dr. T. H. Davies in their work and for the many helpful discussions with Drs. A. J. Cohen, J. A. Laswick and N. H. Sloane. The assistance of Miss L. Boggs is greatly appreciated.

The various materials used in these experiments were donated by the following individuals: Fractions of human plasma by Dr. J. H. Hink, Cutter Laboratories and by Dr. R. B. Pennell, Protein Foundation, Inc.; JB516 and its derivatives by Professor J. P. Buckley, University of Pittsburgh and by Drs. J. H. Biel and H. L. Friedman, Lakeside Laboratories; EDTA, its derivatives and the technical data by Dr. H. F. Harrison, Geigy Industrial Chemicals.

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Biochemical Pharmacology, 1960, Vol. 5, pp. 187–191. Pergamon Press Ltd., Printed in Great Britain.

THE IMPORTANCE OF THE N-TERMINAL END OF ANGIOTENSIN II FOR ITS PRESSOR AND OXYTOCIC ACTIVITIES*

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(*Received* 6 *May* 1960)

Abstract—The importance of the N-terminal end of angiotensin II for its biological activity has been investigated by a comparative study of the pressor and oxytocic potencies of Val^5 angiotensin II and the following peptide analogues: (1) Asp(NH_2). Arg.Val.Tyr.Val.His.Pro.Phe; (2) Gly.Arg.Val.Tyr.Val.His.Pro.Phe; (3) Arg. Val.Tyr.Val.His.Pro.Phe; (4) Val.Tyr.Val.His.Pro.Phe. It was found that: (1) the absence of the N-terminal aspartyl residue of angiotensin II resulted in a parallel reduction of the pressor and oxytocic activities to 26 and 23 per cent, respectively; (2) the removal of the N-terminal aspartyl-arginyl portion of angiotensin II abolished the two activities; (3) the blocking of the free γ-carboxyl group of the aspartyl residue by an amide bond caused a greater reduction of oxytocic (73 per cent) than pressor activity (47 per cent).

IN A previous paper[1] we have reported the marked oxytocic activity of synthetic angiotensin II-amide[2] and studied the effect of enzymic and partial acid hydrolysis on the biological activities of the peptide. We have concluded, from such a study, that the C-terminal phenylalanine residue and the N-terminal aspartyl-arginyl moiety of angiotensin II are equally essential for both its pressor and oxytocic activities, but there was reason to believe that the N-terminal aspartyl residue contributed to the two activities to different extents.

In the present communication we report a further study of the relation between the N-terminal end of angiotensin II and its pressor and oxytocic activities, consisting of a comparison of the two activities in the five following peptides:‡ (1) Asp.Arg.Val. Tyr.Val.His.Pro.Phe (angiotensin); (2) Asp(NH_2).Arg.Val.Tyr.Val.His.Pro.Phe (angiotensinamide); (3) Gly.Arg.Val.Tyr.Val.His.Pro.Phe (glycyl octapeptide); (4) Arg.Val.Tyr.Val.His.Pro.Phe (arginyl heptapeptide); (5) Val.Tyr.Val.His. Pro.Phe (valyl hexapeptide).

EXPERIMENTAL

Materials

Val^5 angiotensin II (angiotensin), Val^5 angiotensin II-amide (angiotensinamide) and the octapeptide Gly.Arg.Val.Tyr.Val.His.Pro.Phe (glycyl octapeptide) were synthetic products kindly supplied by Dr. R. Schwyzer, Ciba Limited, Basel. These substances had been purified by counter-current distribution as free peptides, in the

* This work was subsidized by the Rockefeller Foundation, grant RF 58217.
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‡ The peptides are represented with the aid of the customary abbreviations for the amino acid residues,[3] with the N-terminal residues at the left of the sequences as written.

final stage of the synthesis, and contained from one to three molecules of water and from one to two molecules of acetic acid per molecule of peptide; their purity, checked by paper chromatography, was found to be greater than 99 per cent and, in the case of angiotensinamide, contamination by angiotensin was found to be less than 1 per cent.[4] Twice-crystallized trypsin, lot PM629, was acquired from the Worthington Biochemical Corporation, Freehold, New Jersey.

Methods

Trypsin digestion. One milligram of angiotensinamide was dissolved in 1·0 ml of a 0·05 M ammonium acetate–ammonium bicarbonate buffer of pH 7·7, and to this solution were added 0·05 ml of a trypsin solution containing 5 mg of protein per ml. The molar enzyme : substrate ratio was 1 : 90. After 6 hr at room temperature the reaction was stopped by the addition of 0·5 ml of glacial acetic acid and boiling for 5 min. The mixture was thoroughly evaporated in a desiccator and submitted to paper chromatography for the isolation of the valyl hexapeptide.

The phenylisothiocyanate method of Edman[5] for the stepwise degradation of peptides was modified as follows: 1 mg of angiotensinamide was dissolved in 10 ml of a buffer solution of pH 9·7 prepared by adding 0·7 ml of triethylamine to 2·5 ml of 2N acetic acid and diluting with water to 25 ml. One millilitre of a 20% (v/v) solution of phenylisothiocyanate in acetone was added to the angiotensinamide solution and the mixture shaken vigorously for 3 hr at 40 °C. One millilitre of water was added and the mixture was extracted with seven 5-ml portions of redistilled benzene. The aqueous phase was taken to dryness in a desiccator and redissolved in 5 ml of 3N HCl prepared from glass-distilled constant-boiling HCl. An aliquot of this solution was suitably diluted with 3N HCl and its absorption spectrum in the range 230–280 mμ was immediately determined with a Beckman model DU spectrophotometer. The change in the spectrum was followed by making readings at 30-min intervals, and the release of the phenylthiohydantoin was considered completed when a maximum at 265 mμ and a minimum at 245 mμ were attained. The acid solution was extracted with three 10-ml portions of redistilled ethyl acetate and the aqueous phase was taken to dryness in a rotary evaporator. The dry residue was submitted first to paper ionophoresis, then to paper chromatography for the isolation of the arginyl heptapeptide.

Paper ionophoresis[6] was done on Whatman 3MM paper with pyridine acetate buffer of pH 6·4 and a potential gradient of 15 V/cm for 3–4 hr. After the paper had dried, a guiding strip cut from each edge was sprayed with 0·1% alcoholic ninhydrin solution for the localization of the peptides, which were then eluted with water,[7] dried in a desiccator and redissolved in a small volume of water.

Preparative paper chromatography was done on Whatman 3MM filter paper with the solvent mixture butanol-acetic acid-water (63 : 10 : 27, v/v/v). The peptides were localized with ninhydrin on two guiding strips cut from each edge of the chromatogram, and eluted with water. The eluates were evaporated in a desiccator and redissolved in water. Aliquots of these solutions were taken for aminoacid analyses and for determination of biological activity.

Amino acid composition was determined by dissolving the peptide in a small amount of azeotropic HCl (6N); this solution was enclosed, under vacuum, in a vial and heated at 110 °C for 20 hr. The hydrolysate was evaporated to dryness and

chromatographed on Whatman no. 1 filter paper with butanol–acetic acid–water (63 : 10 : 27) simultaneously with several serial dilutions of a standard mixture containing equimolar amounts of the seven amino acids present in angiotensin. The chromatogram was uniformly sprayed with 0·1% ninhydrin in alcohol and colour was developed at 100 °C for 15 min; the spots were excised, eluted with 75% acetone and the optical density of the eluates was determined at 570 mμ (440 mμ for proline) on a Coleman Jr. model 6A spectrophotometer. The readings obtained for the amino acids applied in known concentrations served as standard recovery curves for the quantitative estimation of the amino acids present in the hydrolysates. The maximum error observed in duplicate runs of the standard mixtures was ± 5%

Biological assays

Pressor activity was assayed on the urethane-anaesthetized rat treated with hexamethonium bromide (50 mg/kg body weight intravenously) and maintained on artificial respiration. Injections were made in the external iliac vein and the blood pressure was recorded from the carotid artery with the aid of a mercury manometer of Condon's design.[8] All solutions were made isotonic before injection.

The oxytocic activity[9] was assayed on the isolated rat's uterus preparation: a virgin female albino rat weighing 150–200 g received a subcutaneous injection of stilboestrol (100 μg/kg body weight) and 15–20 hr later was killed by a blow on the head; one uterine horn was removed and suspended in an aerated 2-ml smooth muscle bath containing de Jalon's solution at 28–31 °C. The peptides to be assayed were diluted with de Jalon's solution and added to the bath in volumes not larger than 0·1 ml. The contractions of the uterine horn were recorded on a smoked drum with the aid of an isotonic frontal lever with a load on the uterus of 1·0–1·5 g and a magnification of six times.

The evaluations of pressor and oxytocic potencies of all the peptides were made by four-point assays against standard solutions of synthetic angiotensin: two doses of each substance, bearing the same higher-to-lower-dose ratio, were administered in three or four groups of four doses, each dose being given once in each group. The heights of the recorded rises in blood pressure or uterine contractions were measured and the data were treated in the manner described by Schild[10] for obtaining the potency ratio and its fiducial limits. For each comparison, two or more assays were made and the separate estimates of the logarithm of the relative potency were combined by calculating their "unweighted" mean.[11] The relative pressor or oxytocic potency of each peptide was defined as the activity of one mole of peptide in relation to the value 100 arbitrarily assigned to the activity of one mole of angiotensin.

RESULTS

Isolation of the valyl hexapeptide

Three components were separated by paper chromatography of the tryptic hydrolysate of angiotensinamide (Fig. 1). The R_f, the quantitative amino acid composition and the pressor and oxytocic potencies of component T_2 indicated that it was residual angiotensinamide not attacked by trypsin, amounting to 18 per cent of the total present in the incubation mixture. Peptides T_1 and T_3 were obtained in a molar yield of 72 and 61 per cent, respectively, and the determination of their amino acid composition

showed that T_1 contained only aspartic acid (asparagine) and arginine, and T_3 had all the amino acids of the valyl hexapeptide in the expected molar ratios.

Isolation of the arginyl heptapeptide

Paper ionophoresis of the residue from the aqueous phase of the phenylisothiocyanate degradation of angiotensinamide, yielded one major component that moved towards the cathode with a mobility that was roughly estimated to correspond to 1·5 positive charges, by comparison with the mobility of lysine. Chromatography of

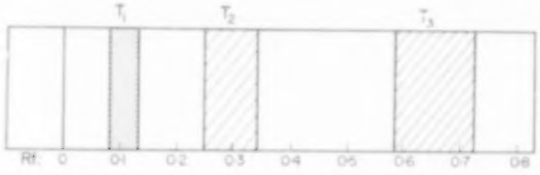

FIG. 1. Diagram showing the resolution of the tryptic hydrolysate of angiotensinamide by paper chromatography. Solvent system, butanol–acetic acid–water (63 : 10 : 27, by volume).

this material with butanol–acetic acid–water yielded a single component with an R_f of 0·42. Hydrolysis and determination of its amino acid composition indicated that it was the expected arginyl heptapeptide, in a molar yield of 17 per cent.

Biological activity

The relative pressor and oxytocic potencies found for the five peptides that were studied are shown on Table 1. The valyl hexapeptide was found to be inactive, in assays that would have detected a relative pressor potency of 1 and a relative oxytocic potency of 0·1. All the other peptides had oxytocic and pressor effects that were qualitatively indistinguishable from those previously described for angiotensinamide.[1] No significant deviation from parallelism between the log dose–response lines for angiotensin and those for each of the different peptides was found in any of the assays ($P > 0·2$). The errors of the biological assays ranged between 5 and 15 per cent at $P = 0·05$.

DISCUSSION

Oxytocic activity

The results of the biological assays of angiotensinamide (Table 1) show that the blocking of the γ-carboxyl group of angiotensin by an amide bond resulted in a decrease of 73 per cent in its oxytocic potency. The same degree of inactivation was observed when the aspartyl residue was substituted by a glycyl residue (78 per cent) or when it was altogether absent (77 per cent). This indicates that the aspartyl residue of angiotensin, though not essential, contributes to a marked degree for its oxytocic activity, and that this is due to the presence of the free γ-carboxyl group.

Pressor activity

Table 1 shows that the removal of the N-terminal residue of angiotensin (arginyl heptapeptide) produced a loss of pressor activity that paralleled that of oxytocic activity. This indicates that the aspartyl group of angiotensin is as important for the pressor as for the oxytocic activity. However, the presence of the free γ-carboxyl

group does not appear to be as necessary for the pressor as it is for the oxytocic activity, since the glycyl octapeptide was a more potent pressor substance than was the arginyl heptapeptide, and angiotensinamide was even more active in that respect.

The ratio of pressor to oxytocic potency was significantly greater for angiotensinamide and for the glycyl octapeptide than it was for angiotensin. This finding suggests

TABLE 1. RELATIVE PRESSOR AND OXYTOCIC POTENCIES OF SYNTHETIC ANGIOTENSIN II AND FOUR PEPTIDE ANALOGUES

		Oxytocic activity*	Pressor activity*
Angiotensin	(Asp.Arg.Val.Tyr.Val.His.Pro.Phe)	100	100
Angiotensinamide	(Asp(NH_2).Arg.Val.Tyr.Val.His.Pro.Phe)	27 (±3)	53 (±4)
Glycyl octapeptide	(Gly.Arg.Val.Tyr.Val.His.Pro.Phe)	22 (±2)	36 (±6)
Arginyl heptapeptide	(Arg.Val.Tyr.Val.His.Pro.Phe)	23 (±3)	26 (±4)
Valyl hexapeptide	(Val.Tyr.Val.His.Pro.Phe)	<0·1	<1

* Pressor and oxytocic activities are expressed by the relative potencies calculated on a molar basis the value 100 being arbitrarily assigned to angiotensin. Figures inside brackets are the fiducial limits at $P = 0{\cdot}05$.

an explanation for the greater persistence of oxytocic, relative to pressor activity, observed[1] during the first hours of partial acid hydrolysis of angiotensinamide: the hydrolysis of the amide bond would unmask the γ-carboxyl group with a consequent increase in oxytocic potency approximately twice as large as the increase in pressor potency.

The absence of detectable pressor and oxytocic activities in the valyl hexapeptide corroborates the previous indications[1, 12] that the aspartyl-arginyl portion of the angiotensin molecule is essential for both activities.

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Biochemical Pharmacology, 1960, Vol. 5, pp. 192–201. Pergamon Press Ltd., Printed in Great Britain.

HAEMATOLOGICAL EFFECTS OF HOMOLOGOUS SERIES OF DICHLOROETHYLARYLAMINES (AROMATIC NITROGEN MUSTARDS)

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(*Received* 28 *May* 1960)

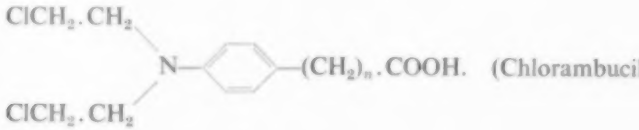
Abstract—The effects of three homologous series of dichloroethylarylamines (aromatic nitrogen mustards): (1) a series of phenylalkanoic acid derivatives, (2) phenoxyalkanoic acid derivatives, and (3) phenyl-α-aminoalkanoic acid derivatives, on the numbers of circulating blood cells and of platelets in the blood of rats have been investigated. Maximum activity in depressing the number of circulating lymphocytes and neutrophils is shown in series (1) by the phenylacetic acid derivative, in (2) by the phenoxypropionic acid derivative, and in (3) by the phenylalanine derivative.

The relation between the haematological activity and chemical reactivity of the compounds is discussed.

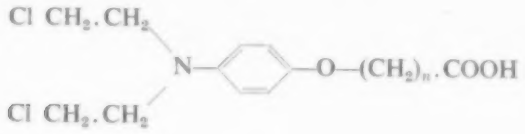
IN DESCRIBING the preparation of aryl-2-halogenoalkylamines ("aromatic nitrogen mustards") Everett *et al.*[1] pointed out that most substances which contain a di-2-chloroethylamine group possessing a certain level of chemical reactivity are cytotoxic to proliferating tissues. Their use as chemotherapeutic agents in neoplastic diseases is limited, however, by the lack in many of them of sufficiently greater specific action on malignant tissue than on some normal proliferating tissues, particularly those of the haemopoietic system. Indeed their therapeutic application has so far shown most promise in the treatment of diseases of this system, particularly the leukaemias.

A large number of "aromatic nitrogen mustards" have been prepared with the object of obtaining more selective action on neoplastic tissue[1–4] and it was often found advantageous to introduce potential water solubility into the molecule by means of the carboxylic acid group (usually solubilized as the Na salt). Chlorambucil (I: $n = 3$) which is widely used therapeutically, particularly in the treatment of chronic lymphatic leukaemia, is a compound of this type. Another compound which is in use therapeutically is the derivative of the naturally occurring amino acid phenylalanine, Merphalan (Sarcolysin)[3, 5] III, $n = 1$. The haematological effects in the normal rat of homologous series of compounds related to these drugs, and also of a series of phenoxyalkanoic acids have now been investigated. These series may be depicted as:

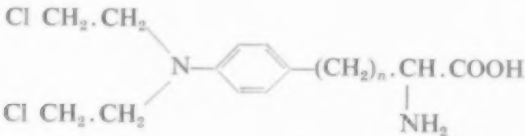
(I) Phenylalkanoic acid series[1]



(II) Phenoxyalkanoic acid series[2]



(III) Phenyl-α-aminoalkanoic acid series[2]



(Merphalan (Sarcolysin): $n = 1$)
(Aminochlorambucil $n = 2$)

In previous work[6] on the effects of a series of compounds related to Myleran which is used in treatment of chronic myeloid leukaemia, it was pointed out that in spite of the difference in pattern of blood counts in the rat compared with the human pattern the effect of the drug on the normal rat can give a reasonable indication of its behaviour in the clinical treatment of leukaemia. The pattern of blood response of the normal rat to a single dose of a chemical has been found of considerable help as a guide in the selection of substances for clinical trial against leukaemia, lymphomas and other neoplastic diseases.[7]

EXPERIMENTAL

Rats of a Wistar albino colony were used. They were kept in separate cages and fed a constant "rat cake" diet during the period of the experiment. Compounds were administered by intraperitoneal injection either dissolved in, or in the form of a fine suspension in, arachis oil.

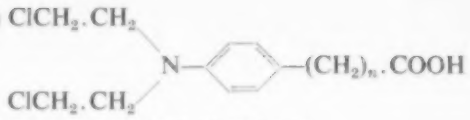
Blood counts

For blood counts, blood was taken from a tail vein direct into the pipette in which it was diluted in the usual manner.

Four rats were usually used for each compound tested and their individual response curves plotted as numbers of blood cells against time. A mean curve giving the percentage of normal count (before injection) against time was then compiled from these individual results.

Toxicity

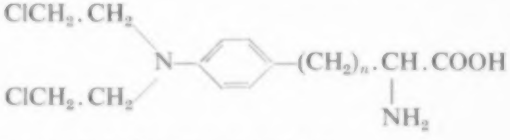
Tests to obtain the approximate toxic dose of the compounds were carried out by intraperitoneal injection in female rats. Groups of three rats were usually used for each dose level tested. The animals were fed a constant "rat cake" diet and weighed daily. The approximate toxicity figures given (Tables 1, 2 and 3) are based on the smallest dose which would kill the three rats tested

TABLE 1. EFFECT OF SERIES (I)  ON THE CIRCULATING LEUCOCYTES IN THE RAT

n	Code no.	Dose (*d*) (mg/kg)	% Fall from normal (*F*) Lymphocytes (*L*)	Neutrophils (*N*)	*L*/*d*	*N*/*d*	Approx. toxic dose (mg/kg)
0	CB 1078	150	33	15	0·2	0·1	>150
1	CB 1331	8 10	90 98	75 85	11·3 9·8 mean 10·5	9·4 8·5 mean 9·0	15
2	CB 1332	16	62	48	3·8	3·0	>20
3	CB 1348	12·5	85	75	6·8	6·0	18
4	CB 1356	20	50	40	2·5	2·0	50

TABLE 2. EFFECT OF SERIES (II) ON THE CIRCULATING LEUCOCYTES IN THE RAT

n	Code no.	Dose (*d*) (mg/kg)	% Fall from normal (*F*) Lymphocytes (*L*)	Neutrophils (*N*)	*L*/*d*	*N*/*d*	Approx. toxic dose (mg/kg)
1	CB 1360	10	50	40	5·0	4·0	30
2	CB 1364	5	70	65	14·0	13·0	10
3	CB 1365	6	60	54	10·0	9·0	14
4	CB 1378	10	80	70	8·0	7·0	16

TABLE 3. EFFECT OF SERIES (III)  ON THE CIRCULATING LEUCOCYTES IN THE RAT

n	Code no.	Dose (*d*) (mg/kg)	% Fall from normal (*F*) Lymphocytes (*L*)	Neutrophils (*N*)	*L*/*d*	*N*/*d*	Approx. toxic dose (mg/kg)
0	CB 1447	20	40	20	2	1	>100
1	CB 3007	10	87	90	8·7	9·0	12
2	CB 1385	12·5	95	55	7·7	4·0	25
3	CB 1494	10	80	45	8·0	4·5	12

RESULTS

Fig. 1 shows the blood response pattern to one member of each of the three series of compounds. In each representative the carboxylic acid group is separated from the benzene ring by the same number of atoms.

The general pattern is the same for all three compounds. For the first few days after treatment there is a fall in all blood components followed by a rapid recovery,

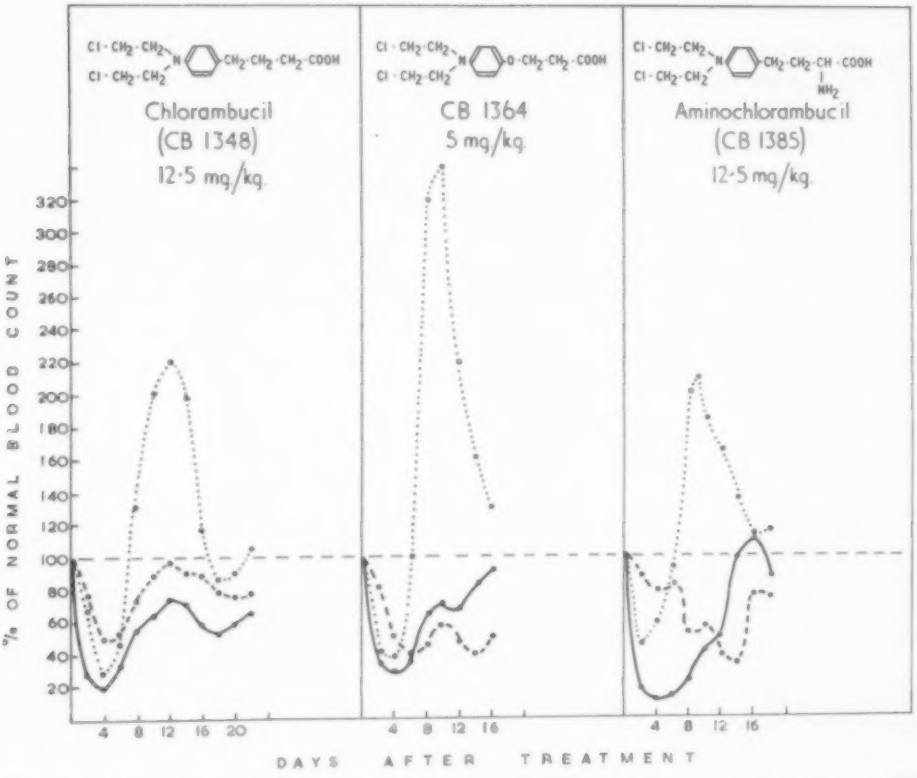


FIG. 1. Blood response patterns of the normal rat to Chlorambucil, 4-N : N-di-(2-chloroethyl) aminophenoxypropionic acid (CB 1364) and Aminochlorambucil. ——— lymphocytes; · · · · neutrophils; — — — platelets.

which in the case of the neutrophils leads to a very large increase in the numbers circulating in the blood, so that there is very marked neutrophilia at about 8–12 days after treatment. The excessive number of circulating neutrophils then falls sharply, and normal values for most blood components are restored by about 20 days after administration of the single dose of the drug.

The relative effects on the two types of leucocytes, viz. lymphocytes and neutrophils, is very similar for Chlorambucil and CB 1364 although with the latter compound only about half the dose is required to produce the same depression. Aminochlorambucil, however, shows a relatively much greater depressive action on lymphocytes than on neutrophils. This more marked action on lymphocytes is also shown by the next higher member of the series, CB 1494 (see Table 3).

Based on these blood response patterns, in an attempt to compare directly the "absolute" activity of the various compounds on the circulating blood cells, an index has been devised consisting of the quotient of the maximum percentage fall from normal values and the dose in mg/kg body weight used to produce this fall. The percentage fall is denoted in the case of the lymphocytes by L, and for the neutrophils by N, the dose being designated d. The index thus becomes L/d, N/d or P/d if platelets are being considered. Although rather crude the index does allow some measure of direct comparison of the absolute haematological activity of different types of compounds. It does not take into account the duration of action in response to a single dose. For instance a compound may produce the maximum fall in lymphocytes in 2, 3, 4 or more days after administration, and this difference is not taken into account in the L/d figure. For true assessment of activity the blood response patterns as shown in Fig. 1 are of course indispensable. Again, the index must of course always be considered together with the toxicity of the compound if it is to be of any use in assessing the therapeutic usefulness of a potential cancer chemotherapeutic agent.

A similar index (F/d) applied only to the neutrophils was used in comparing the haematological activities of the homologous series of compounds related to Busulphan (Myleran).[6] Using the modified nomenclature now described Myleran has an index $L/d = 2{\cdot}3$; $N/d = 8{\cdot}8$. When this is compared with that of Chlorambucil ($L/d = 6{\cdot}8$; $N/d = 6{\cdot}0$) it is seen that the selectively greater effect of Myleran on the myeloid rather than on the lymphoid system is well indicated by the much higher value for the N/d index compared to the L/d value. (This can also be expressed by the low L/N value of 0·26.)

The effects of the three series of compounds (I), (II) and (III) on the circulating leucocytes of the rat are summarised in Tables 1, 2 and 3.

In series (I) (Table 1) maximum activity against both types of leucocytes is shown by the phenylacetic acid derivative (CB 1331: $n = 1$). When $n = 2$ the activity is diminished but returns in Chlorambucil ($n = 3$). The last member of the series ($n = 4$) shows very much reduced activity.

In series (II) the highest activity is shown by the second member (CB 1364: $n = 2$) and then again activity falls rapidly as the value of n increases.

All members of both series show a slightly greater depressive action on lymphocytes than on neutrophils (L/N about 1·2).

In the amino acid series (III) greatest activity against both types of leucocytes is shown by the phenylalanine derivative, Merphalan ($n = 1$). In the higher members of this series, however, activity against lymphocytes does not fall off as in series (I) and (II), although activity against neutrophils does. Thus aminochlorambucil ($n = 2$) is about twice as active in depressing the number of circulating lymphocytes as in depressing the neutrophils ($L/N = 1{\cdot}9$).

The activity against leucocytes of the three series of compounds in relation to their chemical reactivity is illustrated in Fig. 2. The method of assessing chemical reactivity is described in an Appendix by W. C. J. Ross. The very low activities shown by the first members of series (I) and (III) can probably be related to their low chemical reactivity[8] since high biological activity is usually not obtained in compounds which show a percentage hydrolysis of less than 20.[9]

The activity of the most active member of the series (I) and (II) also probably bears some relation to the chemical reactivity. The compound (II-3) is more active than (I-1)

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corresponding with the hydrolysis of 70 per cent against 40 per cent. In the higher members of the series, however, although these all have about the same chemical reactivity, their biological activity appears also to depend on the number of carbon atoms separating the —COOH group from the aromatic nitrogen mustard grouping, activity falling off as this distance is increased.

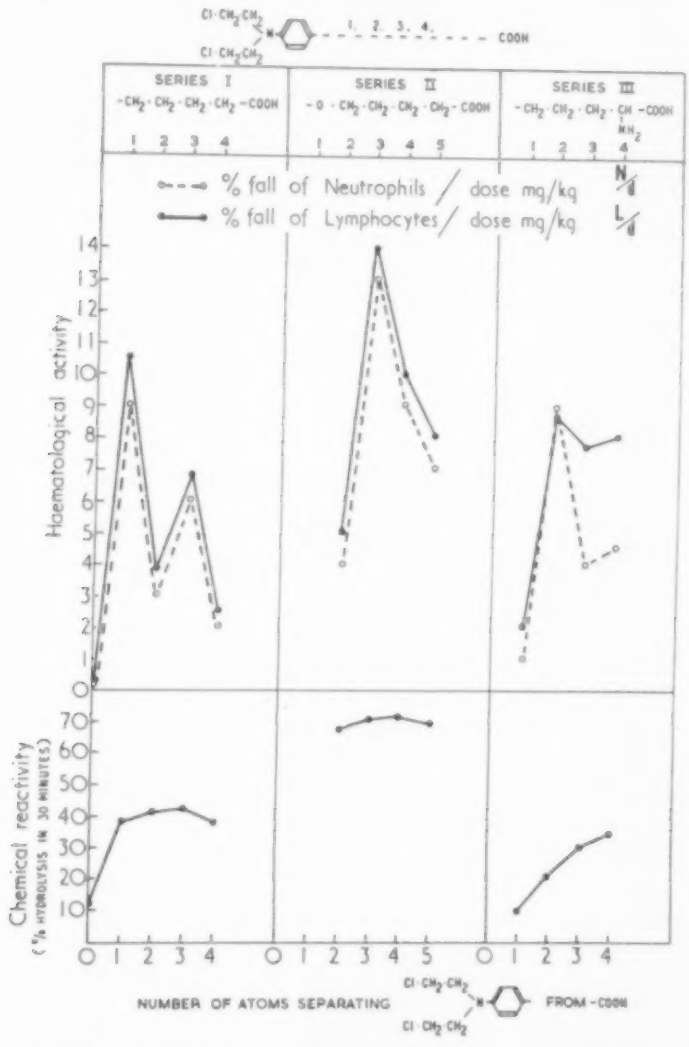
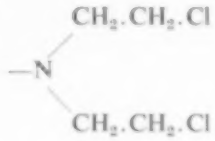


FIG. 2. Haematological activity and chemical reactivity in homologous series of dichloroethylarylamines: (I) phenylalkanoic acid series; (II) phenoxyalkanoic acid series; (III) phenyl-α-aminoalkanoic acid series.

In series (III) although the activity against neutrophils shows a similar falling off in ascending the series, that against lymphocytes remains high. Possibly in this case continuing increase in chemical reactivity compensates to some extent for the fall expected with increasing chain length.

DISCUSSION

In each of the three series of compounds tested the haematological activity of the individual members may be considered in terms of two factors: (a) chemical reactivity of the dichloroethyl amino group—



and (b) the distance of this group from the terminal carboxylic acid (—COOH) group of the compound. It would appear that compounds must have a necessary minimum of chemical reactivity in order to show appreciable biological activity, but once this critical level is surpassed the haematological activity is dependent more on the nature of the molecule as a whole particularly the length of the alkanoic acid side chain.

The toxicity and tumour-inhibitory activity against the Walker rat carcinoma follow much the same pattern as the activity on the blood. In series (I) Chlorambucil was the member originally selected for clinical trials since it appeared to show a rather better inhibitory activity against the Walker carcinoma than the rather more haematologically active CB 1331[1] (Table 1). The most active compound of the phenoxyalkanoic acid series (II) is CB 1364 ($n = 2$). This compound also has a high activity against the transplanted Walker rat carcinoma but is also one of the most toxic.

In series (III) the carbon atom to which the —COOH and $—NH_2$ groups are attached is asymetrically substituted. The compounds described here are all the racemic (DL) forms. The most active compound of this series Merphalan (Sarcolysin) has been resolved into its optical isomers and the L-form, Melphalan, which is thus a derivative of the naturally-occurring L-phenylalanine, is almost twice as active as Merphalan in depressing the circulating leucocytes. The biological action of derivatives of this "L-phenylalanine mustard" Melphalan are described by Elson *et al.*[10]

The most interesting feature of series (III) is the maintenance of high activity against circulating lymphocytes in the higher members of the series. The activity against neutrophils does show a considerable fall in the last two members of the series and thus a compound like aminochlorambucil ($n = 2$) shows a relatively specific action in reducing the numbers of circulating lymphocytes proportionately much more than the number of neutrophils. Elson[11] and Gerhartz[12] have shown that a dose of 12·5 mg/kg aminochlorambucil which results in a greater depression of circulating lymphocytes than the same dose of Chlorambucil causes less damage to the bone marrow. With Chlorambucil there is an 80 per cent depression of granulopoiesis at about 4 days after administration of the drug whereas a much milder depression (40 per cent) occurs with Aminochlorambucil.

In considering the mechanism of action of Chlorambucil and Busulphan, Elson *et al.*[13] considered that whilst Busulphan may exert its biological action mainly by decreasing mitotic frequency, Chlorambucil damages cells about to divide and cells in mitosis. It also damages adult lymphocytes. It would appear probable, therefore, that the greater lymphocyte depressing action of aminochlorambucil may be mainly due to its greater destructive action on the circulating lymphocytes.

Acknowledgements—I wish to record with grateful thanks the participation of my technical assistants, Mrs. J. Black and Mr. A. Coldman, A.I.M.L.T., in this work.

I would also like to thank Mr. J. Everett, F.R.I.C., for preparation of chemicals in suitable form for biological testing. It is also a great pleasure to acknowledge the help received in discussion with Professor A. Haddow, Professor F. Bergel, Dr. W. C. J. Ross and Dr. J. Stock.

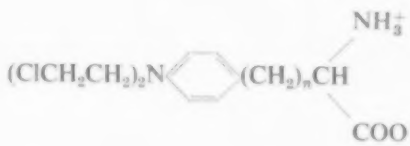
This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research, Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

APPENDIX

*Chemical Reactivity in Relation to Structure of Acid Derivatives of Dichloroethylarylamines**

The relative chemical reactivity of the aromatic nitrogen mustards discussed in this paper may be assessed by determining the rates of hydrolysis under standard conditions. In the examination of a large group of compounds of widely differing solubility it has proved convenient to measure the amount of reaction which occurs when a solution of the compound in 50% aqueous acetone is heated under reflux (66 °C) for ½ hr.[14] The figures obtained do not represent true rate values since no allowance has been made for the pH change during the reaction or for the effect of increasing chloride ion concentration in retarding the rate as time proceeds, though the correction for these factors will be quite small under the conditions chosen. Dr. Davis is at present measuring the rates of reaction of a number of derivatives in purely aqueous solution at constant pH and at high dilution using automatic titration equipment. These new values, which will be reported later, will more closely indicate the rate at which the agents react under physiological conditions. Nevertheless the values obtained using aqueous acetone are a measure of the relative reactivity.

The rates of hydrolysis of the free acidic derivatives and of their sodium salts have been reported.[1, 2] For the present purpose the rates for the sodium salts are the more relevant, for the phenylalkanoic and phenoxyalkanoic acids will be present in the anionic form at physiological pH. In the case of the amino acid derivatives the rate for the free acid will be relevant in the present discussion since under the conditions of hydrolysis and under physiological conditions the compounds will be present as the zwitterion form:



As previously indicated[1] the rate of reaction of a mustard may be followed by measuring the liberation of hydrogen ion or of chloride ion, but in the case of the sodium salts of acidic derivatives the two values are not identical because of a self esterification reaction which will probably not be significant when the agent reacts in a mixed biological system. In a table of reactivities published earlier[9, 15] figures were given for the chloride ion production from phenylalkanoic acid salts

* By W. C. J. Ross, Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, S.W.3.

and for the hydrogen ion production from phenoxyalkanoic acid salts. Whilst these values were adequate for comparing reactivities within a series, it is necessary to consider the chloride ion production alone if compounds in different series are to be compared. Table 4 gives the relevant values for the salts of acids now under consideration.

TABLE 4

Compounds	% liberation of chloride ion in ½ hr. in 50% aqueous acetone at 66 °C from the sodium salt
$(ClCH_2CH_2)_2N$–C$_6$H$_4$–$(CH_2)_n$COOH (I)	
$n = 0$	15
1	39
2	41
3	42
4	39
$(ClCH_2CH_2)_2N$–C$_6$H$_4$–O$(CH_2)_n$COOH (II)	
$n = 1$	68
2	70
3	71
4	70

In the phenylalkanoic acid series the proximity of the carboxylate ion to the benzene ring in the *p*-aminobenzoic acid derivative (I, $n = 0$) has the expected effect of slightly lowering the chemical reactivity as compared with the unsubstituted aniline derivative (hydrolysis under standard conditions, 20 per cent[8]). When this ionized group is separated from the ring by a methylene chain all subsequent compounds have reactivity comparable to that of the *p*-toluidine "mustard" (hydrolysis, 38 per cent).[8] The carboxylate group in the phenoxyalkanoic acid series is sufficiently far removed to have no appreciable effect on reactivity and all compounds have comparable reactivity of the same order as that of the *p*-anisidine "mustard" (hydrolysis, 58 per cent[8]). The greater reactivity of acids (II) as compared with acids (I) is a consequence of the greater electron releasing capacity of an oxygen atom as compared with a methylene group.

Table 5 shows the rates of hydrolysis of the amino acid mustards.

TABLE 5

Compound	% liberation of chloride ion in ½ hr in 50% aqueous acetone at 66 °C from the free acid
$(ClCH_2CH_2)_2N$–C$_6$H$_4$–$(CH_2)_n$CH(NH_2)COOH	
$n = 0$	10
1	22
2	30
3	34

The predominating influence of the positive charge on the nitrogen atom in the zwitterionic form of the amino acid derivative has the effect of reducing chemical reactivity and this effect falls off more slowly with distance than does the effect of a carboxylate ion. A similar gradual falling off of the effect of a charged nitrogen atom has been observed[16] in the direction of nitration of a series of aromatic amines of structure:

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EXCRETION OF 6-AZAURACIL RIBOSIDE IN CANCER PATIENTS

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(*Received* 8 *July* 1960)

Abstract—The writers studied excretion and retention of 6-azauracil riboside after a single dose of 1 g in thirteen different types of malignant and three non-tumorous diseases, altogether twenty-one cases. In four cases, the excretion was followed for a prolonged period of time. In about 50 per cent of the cases there was a total, or nearly total, excretion of unmetabolized 6-azauracil riboside. In the remaining 50 per cent, partial retention and metabolization occurred. In several cases also, deribosidation of 6-azauracil riboside was noted. In cases with prolonged 6-azauracil riboside application, no therapeutic effect on the basic malignant process could be observed. The drug was well tolerated by all patients, and not even during prolonged administration were there toxic or other side-effects noted.

In some experimental tumours, 6-azauracil riboside (6-AzUR) proved to be incorporated in tumour tissue in greater quantities and more selectively than 6-azauracil (6-AzU) alone.[1] According to experimental results, its cytostatic effect is about ten to twenty times greater than the effect of 6-AzU.[2, 3] At the same time, 6-AzUR has a lesser affinity for the central nervous system than 6-AzU, which makes it more suitable for clinical application.[1] In clinical application of 6-AzU, there appear, in about one-third of cases, symptoms on the part of the central nervous system, which render its further or prolonged administration impossible.[4]

When clinically testing the efficiency of a new cytostatic compound the usual procedure is that it is administered in such cases where there is no longer a danger that another, more efficient therapy might be missed, i.e. in advanced cases. However, the advanced stage of a process by itself and—as it is usual in such a case—foregoing treatments are not a suitable ground for the testing of a new cytostatic drug, quite apart from the fact that the evaluation requires a comparatively long time.

6-AzUR lends itself readily to chromatographic isolation and determination. We availed ourselves of this opportunity to obtain information about its retention by the human organism. In so doing we started from the idea that, should this cytostatic be effective also in human tumours according to a similar or identical mechanism, as supposed by Škoda and Šorm,[5] it is necessary that:

(1) the balance of the 6-AzUR administered should be negative; and

(2) it should be more negative in patients with malignant disease than in healthy individuals or patients with non-tumorous disease.

EXPERIMENTAL SECTION

Patients were given a purine-free diet. Three days before administration of 6-AzUR, samples of whole-day urine were chromatographed, according to a method described below, to make sure that the patient did not excrete any substances that might interfere with 6-AzUR determination. On the fourth day, the patient received 1·0 g of 6-AzUR intramuscularly, and we followed its excretion daily until its disappearence, or at least for 3 days.

Chromatographic determination of 6-AzUR was carried out photometrically from the eluates of spots at 258 mμ, calculated[5] from $\epsilon = 6{\cdot}2 \times 10^3$. Chromatography was performed in butanol–water system on Whatman paper no. 4. In the urine in which 6-AzUR determination was carried on, purine bases were precipitated before chromatography and the urine was deprived of salt according to the following method, developed by us:

$$1 \text{ ml urine} + 1 \text{ ml } 10\% \ AgNO_3 + 1 \text{ ml } 0{\cdot}1 \text{ N HCl}$$

In this reaction, chloride ions and purine bases are precipitated, while 6-AzUR remains soluble in the acid solution. Subsequently, the precipitate was filtered and residual $AgNO_3$ in the filtrate was removed by addition of an equal volume of 1 N HCl to the filtrate. After filtration of the AgCl precipitate, 0·2 ml of filtrate was subjected to chromatography.

By the method of photometric determination of 6-AzUR from chromatograms, the following values were obtained:

(1) Determination of 6-AzUR from the aqueous solution, chromatographed directly: spread on the spot: 200 μg; average of determinations: 185 μg; σ of single determination $= \pm$ 10 μg (sixty-four determinations).

(2) Determination of 6-AzUR from the aqueous solution, treated before chromatography according to the above mentioned method for urine: spread on the spot: 133·3 μg; average of determinations: 97 μg; σ of single determination: $\pm$ 13 μg (thirty-five determinations).

(3) Determination of 6-AzUR added to urine, treated according to the above method: spread on the spot: 66·7 μg; average of determinations: 55 μg; σ of single determination: $\pm$ 5 μg (seven determinations).

RESULTS

Results of a single test as to the retained amount of 6-AzUR in twelve different types of tumorous diseases (altogether eighteen patients) and in three patients with non-tumorous disease are shown in Table 1.

As it appears from Table 1, only in two patients with lymphogranulomatosis was the total amount of 6-AzUR retained for a period of 3 days following administration. Seven patients excreted nearly all of the 6-AzUR administered within 24–72 hr after administration. If we take the fact into consideration that, according to our method of determination, lower values were generally obtained (see the Experimental section), we feel justified in supposing that, in about 50 per cent of the patients under test, 6-AzUR was not retained at all.

In four patients (nos. 4, 12, 13 and 18 in Table 1), 6-AzUR was applied for a longer time, excretion being ascertained daily.

In the first of these four patients (no. 4), 6-AzUR was administered for a period of 22 days, namely the first 7 days 2 g daily, then 9 days 5 g and the remaining 6 days again 2 g daily. In the course of application, spots with R_f in the region of 6-azauracil and with an absorption curve similar to that of 6-AzU in the region 220–300 mμ appeared in chromatograms eight times in irregular intervals. After application of a

Table 1. Amount of 6-AzUR retained (dose given 1 g)

Diagnosis	Retained amount (g)
(1) Lymphogranuloma malignum*	1·0
(2) Lymphogranuloma malignum†	0
(3) Lymphogranuloma malignum susp.‡	1·0
(4) Carcinoma pulmonum	0·75
(5) Carcinoma pulmonum	0·3–0·5
(6) Carcinoma pulmonum	0
(7) Leukaemia acuta	0
(8) Leukaemia acuta	0·83
(9) Leukaemia myeloica chronica	0·70
(10) Leukaemia lymphatica chronica	0·45
(11) Neoplasma mediastini	0
(12) Carcinoma mammae, metast. ad pulmo	0·1
(13) Neoplasma renis	0·46
(14) Carcinoma ventriculi	0·15
(15) Carcinoma cutis	0·65
(16) Carcinoma cutis	0·0–0·2
(17) Sarcoma regionis supraclavicularis	0·8
(18) Melanoma malignum	0·4
(19) Ulcus ventriculi	0
(20) Diverticulum oesophagi	0·24
(21) Panmyelopathia	0

* Histologically proved, within three years two courses of HN3, a short time ago sudden death due to cerebral tumour, no lymphogranuloma could be proved by biopsy.

† Clinically typical, histologically proved lymphogranuloma.

‡ Clinically and histologically suspect lymphogranuloma, now being followed up, without symptoms.

single dose of 1·0 g, this patient retained about 0·75 g during a period of 72 hr. When given a daily dose of 2 g, he retained about 0·6 g daily and, after administration of a daily dose of 5 g, he retained on an average 0·77 g daily. In this case, retention of 6-AzUR was not proportional to the increasing dose of 6-AzUR.

The second patient (no. 12), who, after a single dose, retained 0·1 g, received 2 g of 6-AzUR for a period of 13 days and retained 0·2 g on an average.

The third patient (no. 13), who after the first administration of a single dose retained 0·46 g, was given 2 g daily for a period of 11 days, of which he retained daily 0·8 g on an average.

The fourth patient (no. 18), who, after single application of the drug, retained 0·4 g, was given a daily dose of 2 g for 9 days, retaining on an average 0·6 g per day.

In three of these patients (nos. 4, 12 and 13), Endoxan was administered simultaneously. We did not, however, see any therapeutic effects either at the time immediately after administration of the two drugs or during the later course of the disease.

In none of these four patients, who received 6-AzUR for a prolonged period of time, did we note any toxic or other side-effects during the time of administration

(patient no. 4 was given a total dose of 72 g). The drug was well tolerated by all patients. There was only marked local pain during intramuscular injection; 6-AzUR was therefore administered together with procain.

DISCUSSION AND CONCLUSIONS

In the above tests of the balance of 6-AzUR administered to individuals, the drug passed unmetabolized out of the body in about 50 per cent of the cases. In the remaining 50 per cent, it was retained to a certain extent, but also in these cases, as far as it was excreted, it remained unmetabolized. Only three cases were strongly suggestive of the fact that it became partially deribozidated, since in the chromatograms there appeared spots with R_f and extinction curve characteristic of 6-azauracil.

There was no difference in the degree of 6-AzUR retention between the patients with malignant disease and the three patients with other diseases. It is perhaps interesting to note that, from three cases of lymphogranuloma under investigation, in one instance the whole amount of 6-AzUR was excreted, while in two instances all of it was retained or became metabolized. We feel inclined to believe, however, that excretion and/or retention of 6-AzUR are subject to fluctuations, since in patients where we studied excretion from day to day for a prolonged period of time, comparatively great fluctuations in the amount excreted were noted, but no instance of total retention.

We endeavoured to include in our series, cases with different types of malignant disease. In doing so we wanted to ascertain differences, if any, in 6-AzUR retention between single types of tumours. The response of single types of tumour to a given cytostatic differs greatly, and is also often different in one and the same type of malignant disease. We were therefore anxious to include in our first investigation a somewhat wider range of tumours. However, with the exception of the remarkable instance of total 6-AzUR retention in two cases of lymphogranuloma, it does not seem to us that we could draw further conclusions from the other types of tumorous disease included in the present series. It is also possible that the method employed by us for the determination of 6-AzUR excretion is not accurate enough to ascertain minuter changes in the excretion which would be necessary to answer the question that we put to ourselves in the present experiment.

In most cases, after repeated doses the amount of 6-AzUR retained by the organism did not exceed a certain limit. This finding might be of significance when one goes deeper into the question of therapeutic utilization of this drug. We believe that, in order to obtain such levels of 6-AzUR in tumour tissue as might be required for the inhibition of metabolic processes of nucleic acids, it is necessary to search for new forms of application (administration at shorter intervals, local application, chemical substitution of 6-AzUR leading to increased retention).

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MUTAGENIC EFFECTS OF NITROGEN MUSTARD DERIVATIVES OF AZO-BENZENE COMPOUNDS IN *DROSOPHILA MELANOGASTER*

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(*Received* 8 *July* 1960)

Abstract—The azo-mustard 4-di-2″-chloroethylaminoazobenzene-2′-carboxylic acid was found to be mutagenic in *Drosophila melanogaster* males. The mutagenic effect was found only in pre-meiotic germ cell stages and, of these, late spermatids were the most sensitive. Sterility or egg unhatchability was found to be due to aspermia. Within the germ cell stage sterility was found to be correlated to the mutagenic effect suggesting that the differential response of germ cell stages to the mutagenic action of the mustard was not a genetic phenomenon. The induction of sex-linked recessive lethal mutation was found to be linearly related to dose.

The rate of excretion of the azo-mustard was determined by a spectrophotometric method. At least 90 per cent of the injected material appeared to be excreted in an unreduced form, in contrast to the expected mode of action of the compound which required prior reduction of the azo-bond.

A further member of the azo-mustard series, its non-mustard homologue and a possible reduction product, were also tested for mutagenic action. Only the former was shown to be mutagenic.

These results are discussed in relation to the hypothesis that reduction of the azo-linkage is a necessary prerequisite for the mutagenic activity of the azo-mustards.

INTRODUCTION

ATTEMPTS to discover chemical mutagens extend back to shortly after the re-discovery of Mendel's Laws. These early studies were of little value[1] and no conclusively positive results were obtained until the discovery, in *Drosophila*,[2] of the mutagenic effects of mustard gas ($S(CH_2CH_2Cl)_2$). Further discoveries of other chemical mutagens arose independently.[3, 4] The field rapidly expanded and a wide range of chemicals, from inorganic salts to complex organic molecules like the alkaloids and purine derivatives, is now known to be mutagenic in a wide diversity of organisms.

The discovery of the ability of certain chemicals to produce mutations led, initially, to the hope that information on the structure of the gene might be obtained by an analysis, akin to formal chemistry, of the reaction of mutagens with possible gene structures.[5] But while the chemistry of these agents has been widely studied, particularly with respect to their pharmacological properties,[6] there has as yet been no link-up

* This work represents part of the studies presented as a Thesis for the degree of Ph.D. in the University of London and was done under tenure of a Gordon Jacob Fellowship of the Royal Marsden Hospital.

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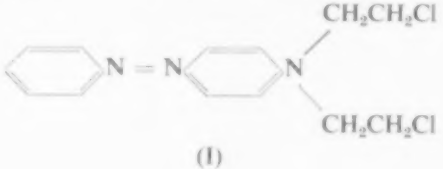
with the recent intensive work on deoxyribosenucleic acid, now widely regarded as the genic material. Furthermore, the ever increasing diversity of chemicals possessing mutagenic properties has raised the problem of whether or not their action can be interpreted as a specific and direct chemical reaction with the chromosomal material.

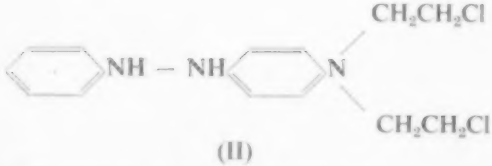
The most common method of approach for the resolution of the above problem has been to compare the effects of different agents. While many differences of effect have been demonstrated, in no case has it been established that the cause of the difference lies in the primary mutagenic action of the various agents. In a recent review, Auerbach[7] has described some secondary effects which might give rise to the observed differences between such mutagenic agents.

Since the early days of radiation mutagenesis, it has been known that different cell types vary in their sensitivity to the mutagenic action of radiation. This principle was also found to hold in chemical mutagenesis and, in addition, it was found that the pattern of genetic sensitivity depended upon whatever chemical agent was used and, in some cases, how it was used. Such variations were principally detected in the various germ cell stages present in the testis of *Drosophila*. The advantage of the *Drosophila* testis was that it contained all stages of spermatogenesis and that the sperm used in successive matings came from successively earlier stages relative to the time of treatment. A sequence of broods could be obtained by mating treated males to fresh samples of virgin females at given intervals and the mutation frequencies observed in these broods would then be an indication of the sensitivity of certain germ cell stages.

The present study was undertaken to examine the brood sensitivity pattern produced by nitrogen mustard derivatives of azo-benzene. These compounds, derived from 4-bis-β-chloroethylaminoazobenzene (I), were synthesized in the expectation that they would be chemically inert,[8] the chlorine atoms of the mustard group having a low reactivity as a result of the powerful electrophilic properties of the azo group. It was thought probable, however, that enzymic reduction of the azo-linkage, if it occurred, would produce the highly reactive hydrazo (II) and amino compounds (III).



(I)



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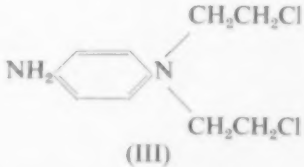


(III)

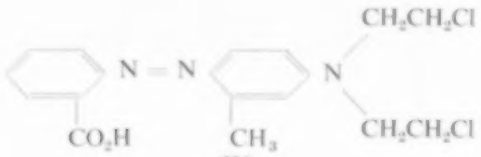
This supposition was supported by the correlation between the cytostatic effects towards the Walker rat carcinoma and the reducibility *in vitro* of compounds of this series.[9]

If, for mutagenesis, these azo-mustards require enzymic reduction, then this might have some influence on their effectiveness within the germ cell stages found in the *Drosophila* testis.

The experiments described below were designed to determine the dose effect in addition to the germ cell stage sensitivity pattern. Data on dose effects in chemical mutagenesis studies are very scanty, due mainly to the difficulty of administering a given dose of mutagen. With the technique reported below this difficulty has been partly overcome.

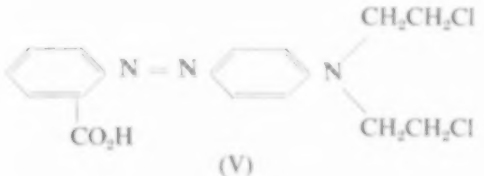
MATERIALS AND METHODS

The compound examined most extensively, and also used in the study of the dose effect, was 4-di-2″-chloroethylamino-2-methylazobenzene-2′-carboxylic acid (IV).



(IV)

Other compounds tested were 4-di-2″-chloroethylaminoazobenzene-2′-carboxylic acid (V), its non-mustard homologue 4-diethylaminoazobenzene-2′-carboxylic acid (VI) and a possible reduction product of IV, 2-methyl-4-N : N-di-2′-chloroethyl-*p*-phenylenediamine (VII).



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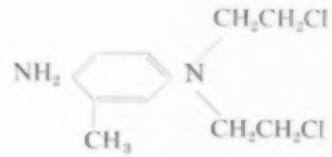
(VI)



(VII)

Compounds were injected into the abdomens of adult male *Drosophila melanogaster* of the Oregon-K strain 48±4 hr after emergence. Compounds (IV), (V) and (VI) were injected as solutions of their sodium salts in 0·4% saline. Compound (VII), which is highly unstable in aqueous solution, was dissolved and injected in arachis oil.

A micrometer syringe ("Agla" Burroughs Wellcome & Co.), equipped with glass needles drawn to a short fine point, was used for the injections. These were done under a stereoscopic microscope at a magnification of about × 20. The syringe was clamped to a stand such that the point of the needle was about ½ mm from the microscope stage. The etherized males, with wing-tips removed, were held with fine forceps and the needle was inserted mid-dorsally between the last two sclerites. The syringe mounting was sufficiently elastic to enable the required movement to be made with

ease. As a check, groups of males were weighed before and after injection. Using the above method, a group of about twenty males could be treated during a single etherization.

In a series of twenty-seven sets of injections, using the above technique, the average weight of aqueous solution injected per fly per unit on the micrometer scale was 0·19 mg; one unit on the scale representing 0·2 μl. Over the twenty-seven observations the correlation between weight of solution injected and the volume as measured on the micrometer scale was 0·98; volumes from 0·1 to 0·6 μl were injected.

The Muller-5 technique[10] was used for the detection of sex-linked mutations and the brood sensitivity pattern was obtained by mating each treated male to two fresh virgin females at 3-day intervals. F_2 cultures were scored initially by observation through the glass wall of the vial using a magnification of $\times$ 20. Cultures suspected of containing a mutation were examined carefully after etherization. This examination was repeated until no further eclosion occurred. The mutations were classed as lethal if no non-Muller-5-males eclosed, or, as visible in the presence of phenotypically distinguishable flies. Mutations were confirmed in an F_3.

Unhatchability tests were made on eggs laid by Oregon-K females inseminated with sperm from treated males, the egg-laying periods of 24 hr being mid-way through the usual brood interval. Unhatched eggs were examined for the presence of dominant lethals after staining by a modified Feulgen technique.[11]

RESULTS

The azo-mustard (IV), like other chemical mutagens and X-rays, produced partial sterility in the treated males, the degree of sterility varying in different broods. Table 1 summarizes the results on sterility brood patterns.

TABLE 1. FERTILITY OF MALES INJECTED WITH THE AZO-MUSTARD (IV) EXPRESSED AS THE AVERAGE NUMBER OF OFFSPRING PER F_0 MALE IN EACH BROOD

Expt.	Brood							Dose mg $\times$ 10^{-5}/fly
	I	II	III	IV	V	VI	VII	
P_ϵ	4·6	3·1	3·0	1·4	9·2	6·4	6·8	32·2
	12·8	11·2	25·0	9·8	40·8	31·8	35·4	16·1
	61·6	53·8	51·0	31·8	29·0	47·4		0·0
P_ζ	38·9	4·1	32·0	50·7				7·8
	26·6	2·9	46·7	36·8				18·7
	24·4	1·8	16·9	21·0				22·6
P_η	53·6	97·0	102·1	105·1	93·6			0·0
P_θ	39·0	27·4	21·4	68·1	33·1	62·7		16·5
	5·4	3·2	0·8	16·7	15·3	69·4		31·4

The sterilizing action of the azo-mustard (IV) was most marked in the first three broods and, of these, brood II was frequently the most sterile. Broods IV and V were almost always highly fertile and in later broods fertility was so high that the majority of F_1 cultures were discarded in order to score the remainder thoroughly.

Table 2 summarizes the data on egg unhatchability. A cytological examination of control unhatched eggs from a different series of experiments showed that 63 per cent of these were fertile when the unhatched frequency was 23 per cent. If it can be assumed

that 63 per cent of the control unhatched eggs were fertile in the present experiment, then the calculated spontaneous contribution to the fertile and non-fertile eggs in the experimental series was twenty-four and fourteen, respectively. Subtracting these estimates from the observed numbers of fertile and non-fertile eggs indicates that in 371 induced unhatched eggs, only twenty-one (5·7 per cent) were fertile and, therefore,

TABLE 2. EGG UNHATCHABILITY INDUCED BY THE AZO-MUSTARD (IV)
(Data from broods I–III. Dose 31·6 × 10^{-5} mg/fly.)

	Experimental			Control
		Cytological examination		
		Fertile	Non-Fertile	
No. eggs laid	7087	—	—	4569
No. unhatched	6197	45	364	377
% unhatched	87·4			8·3
Ca. induced		21	350	

TABLE 3. BROOD MUTATION FREQUENCIES FOLLOWING INJECTION OF THE AZO-MUSTARD (IV)

Expt.		Brood								Dose mg × 10^{-5} fly
		I	II	III	IV	V	VI	VII	VIII	
P_λ	No. tests	327	507	520	345	336				0·0
	No. lethals	1	1	—	—	1				
	%	0·31	0·19	0·0	0·0	0·3				
P_μ	No. tests	5042	3376	5336						3·5
	No. lethals	92	111	87						
	%	1·82	3·26	1·63						
P_ζ	No. tests	930	97	749	449					7·8
	No. lethals	26	2	17	8					
	%	2·8	2·1	2·3	1·8					
P_ϵ	No. tests	501	134	85	62	159	145	102	30	16·1
	No. lethals	27	9	2	—	—	—	—	—	
	%	5·4	6·7	2·4	0·0	0·0	0·0	0·0	0·0	
P_θ	No. tests	656	415	246	589	466	502			16·5
	No. lethals	29	18	10	5	1	—			
	%	4·4	4·3	4·1	0·8	0·2	0·0			
P_λ	No. tests	969	879	661	816	344	361			18·5
	No. lethals	37	38	21	13	—	—			
	%	3·8	4·3	3·2	1·6	0·0	0·0			
P_ζ	No. tests	761	75	845	342					18·7
	No. lethals	30	4	21	5					
	%	4·0	5·3	2·5	1·4					

true dominant lethals. It is obvious that dominant lethality contributed little to the total induced egg unhatchability.

Sex-linked recessive mutation

The data on the induction of sex-linked recessive lethals by IV are summarized in Tables 3 and 4. Irregularities in the number of tests per brood in each experiment

were due to lack of F_1 offspring, conditioned by sterility in the earlier broods, and F_0 male mortality in the later broods. In the majority of experiments the highest mutation frequency was found in brood II, broods I and III were also highly sensitive to the treatment, brood IV was very much less so and in later broods there was no evidence of induced mutation. In all but the experiment with the lowest dose, the differences between mutation frequencies in broods I and II were not statistically significant. The high degree of sterility in brood II made it impossible to put up sufficient tests to obtain an efficient estimate of the mutation frequency. In two experiments using doses of 1·65 and 1·85 mg $\times$ 10^{-4}/fly (Table 3) where large numbers of tests were put up in brood II, mutation frequencies in the first three broods showed little variation. It seems plausible that in these experiments some overlapping occurred so that high sterility and mutagenic sensitivity were shared among the broods.

TABLE 4. BROOD MUTATION FREQUENCIES FOLLOWING INJECTION OF THE AZO-MUSTARD (IV)—(*contd.*)

Expt.		Brood I	II	III	IV	V	VI	VII	VIII	Dose mg $\times$ 10^{-5}/fly
$P\zeta$	No. tests	923	51	340	342					22·6
	No. lethals	45	4	15	2					
	%	4·9	7·9	4·4	0·6					
$P\varepsilon$	No. tests	169	104	410	388					31·2
	No. lethals	16	7	17	4					
	%	9·5	6·7	4·1	1·1					
$P\vartheta$	No. tests	111	3	—	190	231	277			31·4
	No. lethals	12	—	—	—	2	—			
	%	10·8	—	—	0·0	0·9	0·0			
$P\eta$	No. tests	88	40	—	320	106	43			31·6
	No. lethals	4	2	—	4	1	—			
	%	4·6	5·0	—	1·3	0·9	0·0			
$P\iota$	No. tests	174	81	19	82	146	231	48	5	
	No. lethals	11	8	—	—	1	—	—	—	32·2
	%	6·3	9·9	—	0·0	0·7	0·0	0·0	—	
$P\zeta$	No. tests	20	—	35	255					46·2
	No. lethals	—	—	—	3					
	%	—	—	—	1·2					

The experiment at the lowest dose (expt. $P\mu$, Table 3) was designed to clarify the situation with respect to mutagenic sensitivity in the first three broods. The dose of 0·35 mg $\times$ 10^{-4}/fly was chosen in the hope that there would be little sterility in these particular broods. The choice proved successful and there was no lack of offspring in any of the broods. It was decided to make a lower number of tests in brood II on the grounds that if the mutation frequency was highest here, then a greater number of tests in broods I and III would improve the efficiency of the experiment. The total number of tests set up was the maximum that could be handled. In brood II the mutation frequency was almost double that in broods I and III. Comparing the three broods for homogeneity gave a χ^2 of 30 for 2 d.f. ($P < 0{\cdot}0001$). This highly significant result demonstrates conclusively that the germ cells contributing predominantly to brood II were the most sensitive to the mutagenic action of the azo-mustard IV.

The effect of dose

The data on dose effect for sex-linked recessive lethals can be seen in Tables 3 and 4 where the experiments have been listed in order of increasing dose of mutagen. In Fig. 1 the results are represented graphically, the weighted regressions being fitted by maximum likelihood such that $y = a + bx$, where y is the frequency of lethals per cent, and x is the dose of mutagen administered, in mg $\times 10^{-5}$. The parameters, their

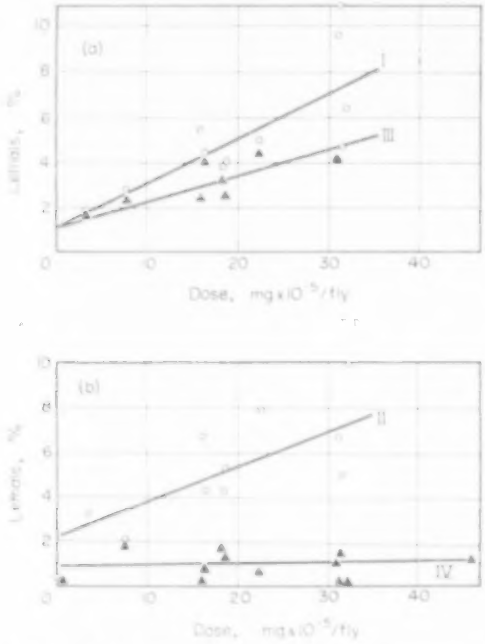


FIG. 1. Dose effect for sex-linked recessive lethals induced by the azo-mustard (IV). Straight lines fitted according to the parameters listed in Table 5. A. Brood I ——— ○. B. Brood II ——— ○. Brood III ——— ▲. Brood IV ——— ▲.

standard errors and a χ^2 for goodness of fit, are included in Table 5. The regression coefficients were highly significant except in brood IV. In this brood the mutation rate was low and the results were not sufficient to supply a precise estimate. The χ^2 for the sum total of broods I–IV showed a highly significant deviation from linearity. This heterogeneity is to be expected because of the variations between broods, with respect to induced mutation rates and sterility. Similarly, in brood II there was a high χ^2 which was just significant at the 5 per cent level. This could be a chance effect but is more probably due to the fact that selective toxicity towards the germ cells was most evident in this brood. In the remaining broods the χ^2 values showed no deviation from the hypothesis of a linear effect. In no instance could a plausible case for a curved regression be put forward. The data are consistent, therefore, with the hypothesis that the mutation frequency increases linearly with dose.

During the above experiments it was noted that the orange-coloured azo-benzene compound could be observed, within 30 min of injection, in the faecal pellets of

treated males. The amount of the azo-compound excreted was, therefore, determined spectrophotometrically. The optical density of a solution of the excreted azo-compound was measured in a Unicam S.P. 500 spectrophotometer, $\lambda = 530$ mμ, using as a blank a similar solution from saline-injected controls. The amount excreted was determined by interpolation, using a graph relating optical density to concentration

TABLE 5. STATISTICAL ANALYSIS OF THE DATA ON DOSE EFFECT WHERE $y = a + bx$; y = FREQUENCY OF LETHALS IN %, x = DOSE IN MG $\times 10^{-5}$/FLY

Brood	a	s.e.	b	s.e.	χ^2	d.f.
I	1·065	0·212	0·198	0·021	12·98	10
II	2·233	0·460	0·157	0·047	14·76*	7
III	1·078	0·190	0·116	0·021	10·10	7
IV	0·906	0·302	0·004	0·014	5·24	5
I–IV	1·338	0·236	0·164	0·025	34·76***	11

as measured with the solution originally made up for the injections. The results of three such experiments (Table 6) showed not only that excretion was rapid, but also that between 90 and 100 per cent of the injected azo-mustard was excreted as an azo-compound. These results were surprising in that the expected mode of action of the azo-mustards involved reduction.

TABLE 6. THREE SEPARATE MEASUREMENTS OF THE AMOUNTS OF INJECTED MUSTARD (IV) EXCRETED BY *Drosophila* MALES

No. males injected	44	58	79
Total weight mustard injected $\times 10^{-4}$ mg	224·6	139·2	157·0
Weight excreted: 0–24 hr	141·5	106·0	158·0
24–48 hr	11·5	6·0	8·0
residue in fly	40·5	30·5	0·0
Total weight excreted + residue	203·5	142·5	164·0
Recovered (%)	90·0	101·3	104·4

The compounds (V), (VI) and (VII) were tested in an attempt to distinguish between the possibilities that the mutagenic action of (IV) was a property of:

(i) The mustard group *per se*.
(ii) The azobenzene group *per se*.
(iii) The mustard group when activated through reduction of the azo-linkage; the reduction occurring: (a) in the germ cells affected, or (b) at other sites, followed by diffusion of the products into the germ cells.

The results of these mutation experiments are presented in Table 7; the average dose per fly for each compound is included in the last column.

The non-mustard azo-compound (VI) showed no mutagenic activity, while its mustard derivative (V) produced a highly significant increase in mutation frequency at an equivalent molar dose.

The reduction product (VII), injected in arachis oil, likewise showed no mutagenic activity. A similarly non-toxic dose of (IV) had given a mutation frequency of 2·1 per cent (Table 3, $P\mu$). If one assumes: (a) a linear relationship between dose of mutagen and mutation frequency; (b) that 10 per cent of the injected azo-compound was

TABLE 7. BROOD MUTATION FREQUENCIES FOLLOWING INJECTION INTO ADULT *Drosophila melanogaster* MALES OF *A*, *B* AND *C*

Brood	I	II	III	IV	Totals I–IV	Dose mg × 10^{-5}/fly
A. 4-diethylaminoazobenzene-2′-carboxylic acid (VI)						
Chromosomes tested	739	738	735	712	2924	
Lethals	3	2	—	2	7	21·0
% lethals	0·41	0·27	0·00	0·28	0·24	
B. 4-di-2″-chloroethylaminoazobenzene-2′-carboxylic acid (V)						
Chromosomes tested	930	874	892	906	3602	
Lethals	33	23	20	18	94	19·4
% lethals	3·55	2·63	2·24	1·99	2·61	
C. 2-methyl-4-N : N-di-2′-chloroethyl-p-phenylenediamine (VII)						
Chromosomes tested	722	715	749	560	2746	
Lethals	2	2	—	1	5	2·6
% lethals	0·28	0·28	0·00	0·18	0·18	

reduced to the amine VII; and (c) that the amine was the active agent, the molar dose of the amine would be equivalent to a molar dose of (IV) that produced 15·6 per cent sex-linked recessive lethals. This expectation must be modified, however, since the amine was administered in arachis oil, and under such conditions mutagens are generally only about one-tenth as efficient as when they are administered in aqueous solution.[12] Thus a mutation frequency of about 1·6 per cent would be expected. The observed frequency was only 0·18 per cent ± 0·17, this is significantly lower than expectation and does not differ significantly from the spontaneous mutation frequency which, in the stock used, was about 0·3 per cent.

DISCUSSION

In the testes of newly hatched male *Drosophila* all germ cell stages up to non-motile spermatozoa are present. The immature spermatozoa mature rapidly and, at the time of treatment (48 ± 4 hr after emergence) cell stages from spermatogonia to fully-mature spermatozoa are represented.

Information on the identity of germ cell stages sampled in successive broods of offspring from treated males is somewhat contradictory. The significant part of the controversy concerns the position of that brood in which the sperm is derived from cells undergoing meiosis at the time of treatment. Some observations on the induction of dominant lethals by X-rays have suggested that meiotic cells are sampled in brood IV[13] and others, of a similar nature, suggested brood III.[14, 15] Histological studies on the testes of males of the wild type stock used in the present work suggested, however, that meiosis is sampled in brood V.[16] This conclusion being supported by a study of germinal selection in the brood sequence[17] and by an analysis of autosomal lethal mutation clusters.[18] Clusters were frequent in broods VI onwards, but absent from the earlier broods. Such clusters arise when mutations are induced in dividing spermatogonia.

For the interpretation of the brood pattern produced by the azo-mustard (IV), it is immaterial whether meiosis is related to brood IV or V. In either case the mutagen was highly effective towards spermiogenic stages and, except for a very low mutagenic effect in brood IV, ineffective in the earlier stages of spermatogenesis. The stage most sensitive to the mutagenic activity of the azo-mustard was that sampled in brood II, probably late spermatids. Both mature spermatozoa and early spermatids were somewhat less sensitive.

The genetic sensitivity pattern was closely paralleled by the toxic effect of the treatment as measured by egg unhatchability or sterility. The induced egg unhatchability was due chiefly to the absence of sperm and since quite drastic chromosomal aberrations do not render sperm inviable, it would appear that the sterility effect was a general cellular toxicity and not a genetic phenomenon. In this case, the close parallel of genetic and toxic effects would suggest that they are both determined by some general aspect of the treatment such as penetration of the mutagen into the cell. The different degrees of genetic sensitivity of the germ cell stages, the brood pattern, were merely the response of the chromosomes in the respective cell types to different concentrations of the mutagen. A similar parallel was found between the mutation and the sterility brood patterns for another class of chemical mutagens (amino acid mustard[19]) in which the actual pattern differed from that above.

Dose effect

Analysis of the results with respect to individual broods showed that, for the azo-mustard (IV), the mutation rate was constant over the dose range 0·0 to $32{\cdot}3 \times 10^{-5}$ mg/fly (Table 5).

A linear dose–mutation frequency relationship has been described for triethylene-iminotriazine.[16, 20] This was based on total mutation frequencies over a series of broods. The interpretation of this relationship is, however, complicated by the fact that the broods sampled were markedly heterogeneous with respect to the induced mutation frequency and sterility and that the number of broods included in the sample varied at different doses. In particular the higher doses were tested only over the first four broods, the lower doses[16] were tested in up to seven broods.

Muller[21] reviewed the problems associated with the linear dose-relationship in radiation genetics with particular respect to the "target" theory. One major objection to this theory was that radiations may produce some of their effect through the formation of a chemical intermediate, since certain irradiated materials proved mutagenic

to bacteria.[22] The results of the present study lend some support to this objection, since the formation of a chemical intermediate need have no marked effect on the rate of increase of mutation frequency with respect to dose, provided that the production of the chemical intermediate is itself linearly related to the dose of radiation given. This latter point is difficult to demonstrate in living cells. Peroxides, however, have been closely linked with the genetical effects of radiation and it is significant that, at least at low doses, the production of H_2O_2 in irradiated water is linearly related to dose.[23] A linear relationship between dose and effect for nitrogen mustards, however, cannot be extrapolated to include other mutagens like peroxides. A conclusive proof of the above point would require an exhaustive knowledge of the chemical changes induced in the cell by radiation, and of the quantitative mutagenic effects of the possible secondary mutagens.

A further criticism of the "target" theory concerns the dose effect on small deficiences. These apparent two-break effects followed a single hit rule:[21] in other words, the mutagenic effect of a single event was spatially separated in the chromosome. One explanation of this anomaly was that the linear dose relationship for the two-break small aberrations arose through the breaking of the chromosome in two places by separate ionizations within the ion cluster of the final track of an ionizing particle,[21] the frequency of these ion clusters being proportioned to dose. It has been shown, however, that the frequency of small deficiencies produced by mustard gas is similar to that produced by X-rays at mutagenically equivalent doses.[25] If the azo-mustards are comparable to mustard gas in their production of small deficiencies, as seems *a priori* probable, then for chemicals also, the frequency of small deficiencies is proportional to dose. Deficiencies form a large part of the sex-linked lethal count (20 per cent) and any increase proportional to the square of the dose should produce a measurable effect on the total lethal frequencies: this was not observed. The hypothesis that small deficiencies induced by radiations are the result of two breaks produced by separate ions in an ion cluster is, therefore, unnecessary since it cannot be argued that the primary mutagenic action for chemicals is similarly non-randomly distributed.

Mode of action of azo-mustards

The azo-mustards differ from previously tested mustard compounds in that the chlorine atoms have a very low reactivity. One hypothesis on their mode of action was that the azo-linkage might be enzymically reduced *in vivo* to produce the highly reactive amine mustards. An alternative hypothesis was that the mustard group was not implicated in the mutagenic action, but that this was a property of the azo-benzene group, the azo-compound 4-dimethylamino-azobenzene (butter yellow) being mutagenic in *Drosophila*.[26] The lack of any measurable mutagenic effect of the non-mustard azo-compound (VI) at a dose at which the mustard homologue (V) produced up to 3·5 per cent sex-linked recessive lethals was, however, conclusive evidence against this alternative hypothesis. The mutagenic properties of the azo-mustards are clearly dependent on the presence of the chloroethylamino group(s).

Two hypotheses have been put forward to explain the mutagenic properties of the mustard group in the above azo-mustards, either that the mustard group may react directly with the genetic material, or that it may require activation by reduction of the azo-bond. We will consider the second of these hypotheses.

If the reduction occurred at sites remote from the germ cells, the highly reactive reduction product would have to diffuse intact, perhaps for a considerable distance. The treatment of flies with a solution of the mustard (V) in arachis oil was an attempt to reproduce this condition. The absence of any mutagenic effect in this experiment is, therefore, evidence against the hypothesis that the mutagenic action of the azo-mustards depends on reduction of the azo-linkage outside the germ cells.

Unlike the inhibition of tumour-growth, which may be due to diverse reactions at various sites, the production of mutations appears to be a specific phenomenon. One must suppose that the mutagenic potentialities of a compound can be realized only if it can penetrate the nucleus in its active form. Thus a highly reactive chemical would appear non-mutagenic because of its inability to reach the chromosomes unchanged. In view of this possibility a simple correlation between chemical reactivity and mutagenicity should not be expected. In fact, such a correlation has not been found except in closely related members of homologous series.[27, 28] In view of this, the failure of the highly reactive reduction product (IV) to produce mutations may be plausibly attributed to an inability to reach the genetic material in sufficient concentration to produce a measurable effect. This interpretation is supported by the observation that less reactive, but otherwise similar, arylhalogenoalkylamines have been shown to be mutagenic in *Drosophila*.[27]

It is necessary, therefore, to suppose that if reduction of the azo-linkage is a prerequisite for the mutagenic action of the azo-mustards, it must take place inside the germ cells and therefore close to the chromosomes. This would require the presence of the appropriate enzymes in the germ cell. Little appears to be known about metabolism in the sperm of insects, but in mammalian sperm the middle cytoplasmic area has been shown to be rich in enzymes.[29] This hypothesis is, therefore, at least plausible. The fact that the fly was able to excrete at least 90 per cent of the azo-compound in an unreduced form, plus the fact that the mutation frequency—dose relationship was linear, suggested, that reduction of the azo-bond was not a necessary prerequisite for its mutagenic action. The brood sensitivity pattern did not seem to be correlated to cell metabolism since the more actively dividing cells proved resistant to the mutagenic action of the azo-mustards. The brood pattern would appear to be determined, in part at least, by secondary characters such as the degree of penetration of the mutagen into the cells, such that this lack of effect in actively metabolizing cells cannot be viewed as evidence against the hypothesis that the azo-mustards exert their mutagenic effect only after intracellular reduction.

A possible explanation of the mutagenic efficiency of the azo-mustard might be that their relatively low reactivity enables them to penetrate the cell easily. Thus the chromosomes might be exposed to a high actual concentration of the compound, whereas, with the more reactive mustards only a very small fraction of the given dose actually reaches the chromosomes. Such an hypothesis, although tentative, would be supported by the linear dose effect and would also explain the excretion of the azo-compound largely unreduced.

Acknowledgements—I wish to thank Dr. G. P. Warwick for his interest in this work and for his advice on the compounds tested. I am also indebted to Dr. J. F. Scott of the Unit of Biometry, Oxford for the statistical analysis of the dose relationship data.

This investigation has been supported by grants to the Chester Beatty Institute (Institute of Cancer Research, Royal Marsden Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

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Vol.
5
1960

Biochemical Pharmacology, 1960, Vol. 5, pp. 219–224. Pergamon Press Ltd., Printed in Great Britain.

INHIBITION OF DEHYDROGENASES OF HUMAN LEUKOCYTES BY HEXETIDINE

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(*Received* 19 *May* 1960)

Abstract—The inhibition of dehydrogenases of human leukocytes was estimated by measuring the percentage of cells which reduced neotetrazolium in the presence of the inhibitor. The mode of inhibition suggests a mechanism involving pyrophosphate-linked coenzymes, divalent cations, and sulfhydryl groups. Dehydrogenase activity was also found to be influenced by a variety of other substrates and inhibitors.

Enzymatic reductions of tetrazolium salts have been shown in a variety of tissues. The activity has been related to DPN-* and TPN-linked flavoproteins called diaphorases,[1] and to the succinic dehydrogenase complex.[2, 3] The ability of human neutrophilic leukocytes to reduce neotetrazolium and thus to demonstrate dehydrogenase activity has been reported by Wachstein,[4] Vercauteren[5] and by DeSouza and Kothare using nitro blue tetrazolium.[6] Neotetrazolium, water soluble and colorless, produced cytoplasmic purple granules (formazan) when incubated with leukocytes, indicative of cytoplasmic sites of reducing activity. Hexetidine, a synthetic antibacterial agent with a broad antibacterial and antifungal spectrum, has previously been shown to inhibit the methylene blue coupled oxygen consumption in human erythrocytes.[7] The inhibition was competitively antagonized by TPN and to a small extent by thiamine pyrophosphate (TPP). In contrast, glycolytic reactions were found to be much less sensitive to the inhibitor. TPP has also been found to be effective in reversing the inhibition of pyruvate oxidation by a particulate fraction of spores of *B. cereus var terminalis*.[8] Hypothetically, therefore, inhibition of respiratory enzymes may be a selective mode of action of hexetidine. In substantiation of this hypothesis, in this report hexetidine was observed to inhibit neotetrazolium-reducing dehydrogenases when incubated with leukocytes, as demonstrated by a diminished number of cells containing microscopically visible cytoplasmic formazan granules. The activity could be restored by DPN and by several divalent cations, notably zinc ions. The effects of a group of substrates and inhibitors on the endogenous reaction were also observed.

EXPERIMENTAL

Collection of cells

Leukocytes were obtained by centrifugation of heparinized human venous blood in Cushman tubes. Two tubes of from 8 to 10 ml capacity provided sufficient cells

*Abbreviations used are: hexetidine (1 : 3-bis-β-ethylhexyl-5-methyl-5-aminohexahydropyrimidine) obtained from the Warner Lambert Research Institute, Morris Plains, New Jersey: DPN, TPN, di- and tri-phosphopyridine nucleotide, respectively; TPP, thiamine pyrophosphate; F-1 : 6-di-P, fructose-1 : 6-diphosphate; F-6-P, fructose-6-phosphate; G-6-P, glucose-6-phosphate.

* Fabian Lionetti unpublished observations.

for a typical experiment. Plasma was aspirated, discarded, and the "buffy coat" of leukocytes extending into the capillary part of the tube carefully removed with a small syringe and a long (no. 15) needle. The cells from 10 ml of blood were suspended in 7·0 ml of 0·11 M phosphate buffer at pH 7·2.

Reagents

Saturated neotetrazolium chloride solution (about 0·3 per cent w/v) was made by adding an excess of the salt to the buffer and shaking mechanically for from 5 to 10 min followed by filtration through medium porosity paper. Substrates, cofactors and inhibitors were also dissolved in buffer to give the concentrations shown in the tables. Hexetidine solutions were prepared as described previously.[7] Phosphorylated carbohydrate esters were obtained as the barium salts, barium ion was removed by treatment with a slight excess of saturated potassium sulfate and the $BaSO_4$ removed by centrifugation. The supernatant solutions were diluted with buffer to yield the concentrations used.

Procedure

The incubation medium contained 3·0 ml of buffered neotetrazolium (0·11 M phosphate pH 7·2), 1·0 ml of leukocyte suspension containing approximately 1×10^7 mixed granulocytes and 2·0 ml of buffer containing various substances in 15·0 ml conical-shaped centrifuge tubes. Controls contained buffer, neotetrazolium and cell suspensions. The total volume was 6·0 ml. Six tubes were used in most experiments and were incubated for 1 hr at 37 °C in a water bath. They were then centrifuged at $500 \times g$ for 10 min and nearly all (about 5·5 ml) of the supernatant solution was decanted. A drop of the concentrated cell suspension at the bottom of the tube was transferred to a slide and mounted as a wet preparation, using a coverslip with greased edges. The slides were further incubated at room temperature for a second hour. They were then observed microscopically ($\times$ 1000) under an oil immersion lens. Of the mature neutrophiles 300–400 were counted and the percentage which contained one or more distinct formazan granules was recorded. Clumped or disrupted cells were not included.

RESULTS AND DISCUSSION

Whereas several di-tetrazolium salts (blue tetrazolium and a nitro derivative, nitro-BT) have been shown to possess advantages for histochemical application,[1, 2] good diformazan deposition was obtained from neotetrazolium with simple media in aerobic incubations. Diformazan granules produced were well formed, very dark and not noticeably diffuse when substrates were present. However, the cytoplasm of the cells always contained sufficient endogenous substrates for a visible reaction. The leukocytes in the control tubes of a large (approximately fifty) number of experiments contained from 30 to 90 per cent of stained neutrophiles, depending on conditions. In a single sample of cell suspension the endogenous reaction could be measured with a precision as indicated by the mean deviation $\pm$ 9·0 per cent. Erythrocytes were unstained. Platelets and lymphocytes, however, did contain visible granules. The endogenous staining reaction could be diminished and occasionally abolished by several conditions. Storage of the cell suspension overnight at 4 °C, or maintaining a sterile whole blood sample at 25 °C for approximately 8–10 hr prior to the collection

and pre-incubation of the cells depleted the endogenous substrate and reduced the production of formazan granules from 50 to 60 per cent. Furthermore, washing the cells three times in isotonic (0·15M) NaCl and then pre-incubating them at 25 °C for 4 hr further reduced the dye reduction, but disruption of much of the cells invalidated quantitation. Whereas the formazan granules in unfortified preparations became diffuse after 2–3 hr and usually disappeared in about 5 hr, those produced when substrates such as succinate were present (Table 1) remained sharp and distinctly visible for 5 hr. The morphological characteristics of the reaction have been well characterized in drawings by Vercauteren.[5]

The persistence of a high endogenous dye reduction defeated most of the attempts to define substrate and cofactor dependencies of the reaction. The cells frequently clumped or lysed when allowed to pre-incubate at 25 °C sufficiently long (4–5 hr) to make the enzyme reaction dependent on exogenous substrate. In spite of this limitation, it was still possible to detect and estimate the effects of a number of substrates and cofactors for dehydrogenases (Table 1). While in some instances noticeable

TABLE 1. EFFECTS OF VARIOUS SUBSTANCES ON DYE REDUCTION IN LEUKOCYTES

Substance added	Number of experiments	Molarity	Mean % change in cells with formazan granules (+ increase, − decrease)
Succinate	6	0·012	+126
Succinate + malonate	5	0·020 0·012	−33
Malate	5	0·018	+3·0
Pyruvate	5	0·019	+5·4
*iso*Citrate	5	0·013	−3·0
Glucose	8	0·018	+51
G-6-P	7	0·016	−5·0
F-6-P	5	0·016	−7·0
F-1 : 6-di-P	8	0·008	−13
Iodoacetate	4	1×10^{-4}	−64
p-Chloromercuribenzoate	2	1×10^{-4}	−57
Fluoride	9	0·020	+73
Versenate (Na_2)	6	0·037	+21
Hexetidine	6	$2·4 \times 10^{-5}$	−55
DPN	5	$1·7 \times 10^{-4}$	−14
TPN	5	$9·0 \times 10^{-5}$	+1·0

The basic system contained only neotetrazolium, phosphate buffer and leukocytes, as described in the text.

changes in the *amount* of formazan pigment per cell were observed with some substrates (glucose, succinate), a regular finding was an increase in the *percentage* of cells containing the formazan. In the case of inhibitors (iodoacetate, hexetidine) a decrease in the number stained was observed. Quantitative estimation of enzyme activity by observing the percentage of cells stained per unit time in a medium of specified composition was therefore possible. Succinate, glucose, fluoride and sodium versenate were observed to stimulate the reaction, whereas iodoacetate, parachloromercuribenzoate, and hexetidine were very effective inhibitors. Malonate, classically known as a competitive inhibitor of succinate, was moderately inhibitory in a ratio

of malonate to succinate of slightly greater than 2 : 1. The other substrates were without effect on the reaction. However, in cells stored for 8 hr at 25 °C prior to incubation with malate (0·012 M) the reaction was much more dependent on exogenous substrate, the stimulation by added malate increasing from a negligible effect of 3·0 per cent (Table 1) to 81 per cent. Neither DPN, TPN nor a TPNH-generating system (glucose-6-phosphate + TPN) produced significant effects on the reaction. In contrast to findings of Antopol *et al.*[9] with fibroblast cells, α-glycerophosphate plus DPN (a potential source of DPNH) had no effect.

Many similarities to the results described by Vercauteren[5] were observed. Although he incubated the cells anaerobically in Thunberg tubes and examined smears fixed in formalin, the endogenous reduction of neotetrazolium (percentage of cells stained) was of similar magnitude to that given here. The only quantitative data for intact cells were obtained with inhibitors present. These data are much like the data obtained here, allowing for certain differences in concentrations of inhibitors. It is inexplicable to us that fluoride (3×10^{-3}) should inhibit Vercauteren's system approximately 30 per cent while at 2×10^{-2} M our reaction was markedly stimulated. With homogenates of leukocytes, however, he found fluoride (3×10^{-3} M) had no effect. Vercauteren has also shown that malate, pyruvate, citrate, and TPN did not affect the reduction of the dye in intact cells. These observations are confirmed in the data of Table 1. Glucose, however, was negative in the cells but stimulated the reaction in

TABLE 2. EFFECT OF HEXETIDINE ON DEHYDROGENASES OF LEUKOCYTES

Tube	Hexetidine (μmoles)	Cells stained (%)
1	0	75
2	0	80
3	0·015	87
4	0·15	50
5	0·38	26
6	0·75	0

Reduction of neotetrazolium was dependent on endogenous substrates, as no substrate was added. Hexetidine is expressed as μmoles per 6 ml of reaction mixture as described in the text.

homogenates. In our intact cell preparation a pronounced stimulation by glucose was observed. Anaerobiasis was not a requirement in our method although the wet mounted preparations were sealed during the final hour of incubation.

The behavior of hexetidine proved interesting in several respects. The various findings with this inhibitor suggested an inhibitory mechanism which operated by interaction with the phosphorylated constituents of coenzymes.[7, 8] Some evidence which partially substantiates this type of mechanism was obtained with the leukocyte neotetrazolium method. The results of an experiment showing an increase in inhibition with increasing concentration of hexetidine are shown in Table 2. This effect could be observed regularly, although the amount of inhibitor necessary to reduce the number of cells containing formazan to 50 per cent varied appreciably in the cells from different donors. However, in most of the experiments described, the amount of hexetidine

necessary for this varied from 0·13 to 0·16 μmoles per tube. Several cofactors were found which could relieve the inhibition if incubated simultaneously with hexetidine and the cells. Although DPN alone exercised no influence on the percentage of cells containing formazan, its presence with hexetidine in molar ratios of 5 : 1 abolished the inhibition. An experiment representative of such findings is shown in Table 3. TPN was without effect. With several notable exceptions cations did not effect the inhibited reaction. Mg^{2+} and Zn^{2+} counteracted the inhibition, while Cu^{2+} was found to add to the inhibition. Zinc ion, however, in the inhibited cell preparation

TABLE 3. ANTAGONISM OF HEXETIDINE BY DPN

Hexetidine (μmoles)	DPN (μmoles)	Cells inhibited (%)
0·15	0	89
0	0	1*
0	1·0	0†
0·15	0·15	53
0·15	0·75	15
0·15	1·0	11

* 99 per cent of cells contained formazan granules.
† 100 per cent of cells contained formazan granules.
Concentrations are in μmoles per 6 ml of reaction mixture.

could repeatedly be shown to reduce the inhibition (Table 4). Other cations in amounts up to five times the amount of hexetidine had no measurable effect on the dye reduction or inhibited reaction. These included NH_4^+, Ca^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+}, and Fe^{3+}.

The relative stimulation of the enzyme reaction with succinate, in contrast to the other intermediates of the tricarboxylic cycle, is interesting in light of the pattern of inhibition displayed. That sulfhydryl active sites are involved is suggested by the high

TABLE 4. PARTIAL ANTAGONISM OF HEXETIDINE INHIBITION BY ZINC ION

Tube	Hexetidine (μmoles)	Zinc ion (μmoles)	Cells stained (%)
1	0	0	94
2	0·15	0	52
3	0·15	0·15	44
4	0·15	0·38	70
5	0·15	0·75	84

The units of concentration are similar to those of Tables 2 and 3.

inhibition shown by 1×10^{-4} iodoacetate and 1×10^{-4} *p*-chloromercuribenzoate. Circumstantially, at least, hexetidine resembles this group. The relative increase in dye reduction by endogenous substrate in the presence of versenate and fluoride suggests that the enzyme reaction is facilitated by complexing interfering cations (possibly Cu^{2+}). The reversal of the inhibition seen with hexetidine by several cations suggests that complexing of ions is one mode in which it might act biologically. In a non-cellular experiment, the formation of a complex of hexetidine and Mn^{2+}, or

Zn^{2+} (between pH 6·0 and 7·5) or Cu^{2+} (between pH 3·0 and 5·0) has been observed potentiometrically.[10] Of these, however, only Zn^{2+} could be shown to reverse the action of the inhibitor biologically. Finally, the reversal of hexetidine action by simultaneous incubation with DPN is consistent with the idea that hexetidine might act by disruption of an active sulfhydryl linked enzyme–ion–coenzyme complex such as that described by Van Eys and Kaplan[11] in explaining why DPN and DPNH competitively antagonize *o*-phenanthroline inhibition of yeast alcohol dehydrogenase.[12] Attempts to show interaction of inhibitor with either DPN or Zn^{2+}, or both, by spectrophotometric methods were unsuccessful. That reduced pyridine nucleotides do not couple directly to neotetrazolium has been shown by Farber *et al.*[1] who attribute the dye reduction to diaphorase enzymes. The leukocyte system, being most responsive to added succinate, may reduce neotetrazolium chiefly through the succinic dehydrogenase complex.

Acknowledgement—We are greatly indebted to Dr. Wm. McNary of the Department of Anatomy of the Boston University School of Medicine for valuable advice on histological techniques.

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Biochemical Pharmacology, 1960, Vol. 5, pp. 225–230. Pergamon Press Ltd., Printed in Great Britain.

PLASMA CONCENTRATION CURVES OF N-METHYLPYRIDINIUM-2-ALDOXIME METHANE SULPHONATE (P2S) AFTER INTRAVENOUS, INTRAMUSCULAR AND ORAL ADMINISTRATION IN MAN

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(*Received* 12 *July* 1960)

Abstract—The rate of absorption of N-methylpyridinium-2-aldoxime methane sulphonate (P2S) after intramuscular and oral administration has been investigated in man.

It is found that 30 mg per kg body weight can be given intramuscularly without serious pain. The rate of absorption varies considerably, however, peak plasma concentrations averaging 15 μg per ml are reached after 20 min and after 90 min the concentrations are about 9 μg per ml. After 5–10 min most of the patients are expected to have therapeutic plasma levels.

Maximum concentration in plasma after oral administration of 45 mg per kg body weight in gelatin capsules is about 5 μg per ml and a concentration over 3·5 μg per ml is maintained from 60 to 240 min. It is considered that oral administration has little therapeutic effect since the rate of absorption is very slow.

After oral administration of 45 mg per kg body weight, 23 per cent of the dose is excreted in the urine within 4 hr.

THE increased use of anticholinesterases of the organophosphorous type as insecticides, during the last 10 years has caused a great number of cases of fatal poisoning.[1–3]

Formerly, atropine was considered to be the most effective drug for counteracting the toxicity of organophosphorous cholinesterase inhibitors, such as parathion, di*iso*propoxyphosphoryl fluoride (DFP), tetraethyl pyrophosphate (TEPP), etc. Recently it has been demonstrated that certain oximes, when used in combination with atropine, provide more effective therapy than atropine alone for animals exposed to these toxic agents.[4, 5] These new drugs reactivate the phosphorylated cholinesterase, and thus provide a causal therapy.[6, 7] One of the most potent oximes in this respect, N-methylpyridinium-2-aldoxime iodide (PAM), has been used in man both in experimental and in accidental organophosphate poisoning.[2, 8] It is claimed to be safely tolerated in man in intravenous doses of 15–30 mg per kg body weight.[9]

Because of its low solubility in water (5 per cent w/v) PAM has only been administered intravenously. N-methylpyridinium-2-aldoxime methane sulphonate (P2S), which is much more soluble (60 per cent w/v), has been given to man orally and by intramuscular injection.[10]

The aim of the present investigation has been to determine the rate of absorption in man of N-methylpyridinium-2-aldoxime methane sulphonate (P2S) after intramuscular injection, and after oral administration. The concentration of P2S in plasma

was therefore determined at certain interval after administration. No analyses were done on whole blood, since P2S has been shown not to enter the erythrocytes of man or dog.[11]

EXPERIMENTAL

The experiments were carried out on healthy volunteers and on patients in the Ear-nose-throat department of Karolinska sjukhuset.* The patients were treated for minor disorders without any evidence of circulatory, gastrointestinal, renal or hepatic disturbances.

TABLE 1. SENSITIVITY AND STANDARD ERROR OF P2S ANALYSES BY HYDROXYLAMINE DETERMINATION
(Method of Askew *et al.*[13])

Concentration in plasma (μg per ml)	Optical density at 555 mμ Mean and standard error of the mean ($n = 9$)
5·0	0·011 ± 0·001
15·0	0·040 ± 0·002
50·0	0·139 ± 0·004

TABLE 2. SENSITIVITY AND STANDARD ERROR OF P2S ANALYSES BY LIGHT ABSORPTION AT 333 mμ
(Method of Creasy and Green[12])

Concentration (μg per ml)	Optical density at 333 mμ Mean and standard error of the mean ($n = 7$)
4·7*	0·088 ± 0·002
9·3*	0·175 ± 0·004
18·6*	0·344 ± 0·004
24·3†	0·465 ± 0·001
27·9*	0·521 ± 0·004
50·6†	0·964 ± 0·005

* In plasma.
† In water.

P2S (synthesized according to the method described by Creasy and Green[12] was Seitz-filtered, freeze-dryed and stored in sterile flasks.† Before use, the content (2·5 g) was dissolved in sterile pyrogen-free water. This solution had a pH of from 3 to 4.

For oral administration, gelatin capsules were filled with 1 g of crystalline P2S.

Blood samples were taken at intervals via a needle in an antecubital vein and collected in heparinized test tubes. A sample was always taken before administration of P2S and enough blood was taken to enable making double tests.

The plasma was stored at − 20 °C till the following day, when it was analysed.

Two methods have been described which are suitable for the determination of P2S in blood and tissues. One is based on the acid hydrolysis of the oxime to hydroxylamine, which is then determined by a colorimetric method,[13] the other is based on

* The patients were medically supervised by H. Diamant, M.D., to whom my thanks are due.
† Dr. R. Barkman, Military Pharmacy, Stockholm 60, has kindly provided the sterile P2S and the P2S capsules.

the ultra-violet absorption spectrum of P2S.[12, 14] The sensitivity and precision of these two methods were determined and the results are summarized in Tables 1 and 2. As the ultra-violet absorption method was the more sensitive one, it was chosen for this investigation.

Two minor modifications were introduced in the original method. The proportions of zinc sulphate to barium hydroxide had to be changed in order to obtain clear solutions, and it was found more convenient to use heparinized plasma instead of whole blood.

Procedure

Two millitres of heparinized plasma were diluted with 3·3 ml water, and 1 ml 0·2 M $Ba(OH)_2$, 1·5 ml 0·22 M $ZnSO_4$ and 0·2 ml of 20% NaCl were added consecutively. After the addition of each reagent the solution was thoroughly shaken. The mixture was then centrifuged for 20 min at 3000 rev/min. To 3 ml of supernatant, 0·2 ml 5 N NaOH was added, and the light-absorption at 333 mμ was determined in a

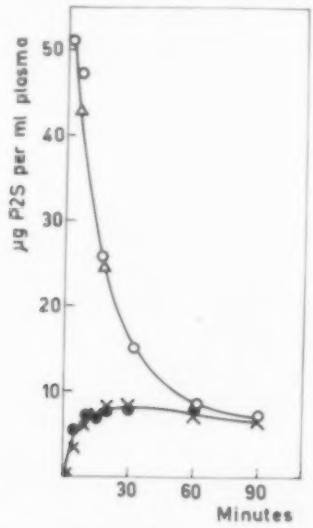

FIG. 1. Plasma concentrations of P2S in man after intravenous and intramuscular injections of 20 mg per kg body weight.
○ Intravenous injection of a 12·5 per cent solution.
△ Intravenous injection of a 25 per cent solution.
× Intramuscular injection of a 12·5 per cent solution.
● Intramuscular injection of a 25 per cent solution.

Beckman DU spectrophotometer in a 1-cm cuvette. The concentration of P2S was read from a calibration curve. The specificity of the method has been studied by Ellin[15] and Ellin and Kondritzer,[14] who found that acid or alkaline hydrolysis of PAM did not produce substances that interfered.

RESULTS

Plasma concentrations of P2S *after intravenous injection*

In preliminary experiments two subjects were injected with P2S (20 mg per kg body weight) by the intravenous route in order to get an idea of the plasma concentrations

after equilibration. The concentrations of P2S in plasma are seen in Fig. 1. The injections caused some mild side reactions such as "dizziness", blurred vision and diplopia, but these symptoms vanished within a few minutes.

Plasma concentrations of P2S *after intramuscular injection*

Two subjects first received 20 mg per kg body weight intramuscularly, in the right gluteal region. The plasma concentrations, as shown in Fig. 1, were found to be almost identical in the two subjects, reaching a peak level of about 8 μg per ml after about 20 min. In one of the subjects a 12·5% solution was used, which caused no pain. The other subject received the same dose in a 25% solution, but this caused some pain at the site of injection.

Ten subjects then received 30 mg P2S per kg body weight, in a 25% solution. Some complained of pain at the site of injection and later of sciatic neuralgia in the leg of

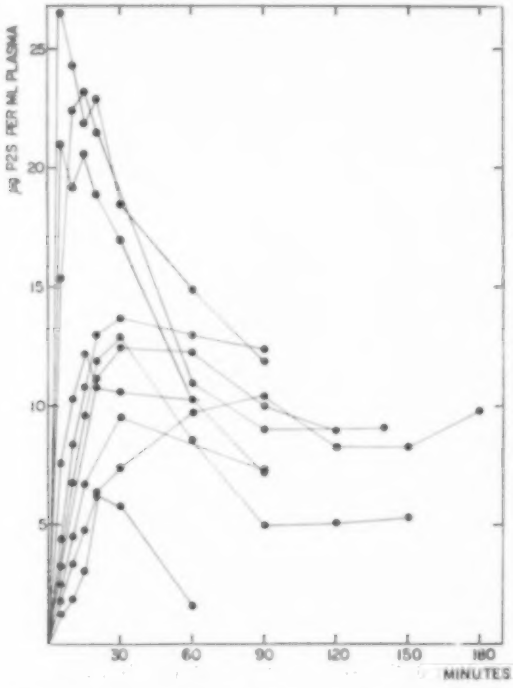

FIG. 2. Plasma concentrations of P2S in man after intramuscular injection of 30 mg per kg body weight.

the injected side, but these symptoms disappeared within a few hours. The plasma concentrations are seen in Fig. 2. A maximum concentration averaging about 15 μg per ml was reached after 20 min and was followed by a progressive fall. The rate of absorption and the maximal plasma levels varied considerably from one subject to another. A plot of the individual results indicated two different patterns of absorption (Fig. 2): one which is fairly rapid with plasma concentrations of about 20 μg per ml after 5 min, and another which is rather slow with plasma concentrations of 10 μg per ml after 20 min.

Plasma levels of P2S *after oral administration*

In these experiments, no food was taken from 5 hr before the start until the end of the experiment. Before the administration of the capsules, 400 ml of water was given orally to facilitate the collection of voided urine samples for the 4 hr period of the experiment.

Three gelatin capsules, each containing 1 g of P2S, were given to each of six subjects, weighing 65–70 kg. The plasma concentrations are shown in Fig. 3. A very slow rise in plasma concentration was observed with a badly defined maximum of from 4 to 5 μg per ml after 2 to 3 hr, followed by a slow decrease.

The amounts of P2S excreted in the urine during the 4 hr after administration averaged 23 per cent of the dose (standard error of the mean $\pm$ 3 per cent).

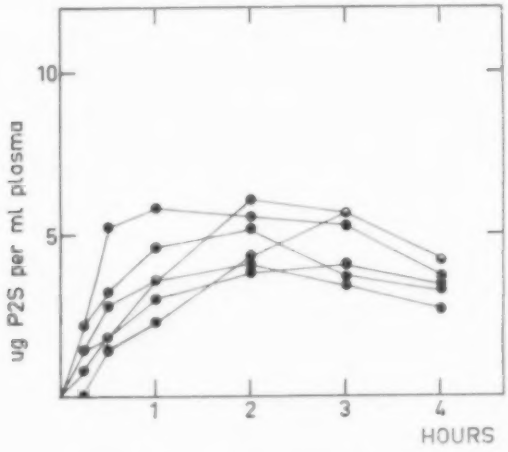

FIG. 3. Plasma concentrations of P2S in man after oral administration of 45 mg per kg body weight in gelatin capsules.

DISCUSSION

The present investigation was carried out in order to find how N-methylpyridinium-2-aldoxime methane sulphonate should be administered in case of organophosphate poisoning.

In mice, rats, guinea-pigs and rabbits the LD_{50} of *iso*propoxymethylphosphoryl fluoride (sarin) is raised by multiples of 4·0, 1·6, 38 and 40, respectively, if 30 mg P2S per kg body weight are given intramuscularly in combination with atropine 1 min after poisoning.[16] It was also stated that little is won in therapeutic effect if the dose of P2S was larger.

The results presented in the present paper show that 30 mg P2S per kg body weight can be given intramuscularly to man without serious discomfort or pain. The rate of absorption, however, varies greatly from subject to subject and this is possibly due to different depths of injection.

Experiments on the anaesthetized cat indicate that plasma levels above 4 μg per ml are needed to counteract the bradycardia and respiratory failure produced by lethal doses of *iso*propoxymethylphosphorylthiocholine.[17] If this figure is valid also in man,

one can assume that therapeutic plasma levels are reached after 5–10 min in most of the cases provided circulatory failure has not yet developed.

The results obtained after oral administration show that a fairly large dose has to be given to obtain effective plasma levels, and these are reached only after a considerable delay. It is then quite evident that oral administration alone is of little therapeutic value as the rate of absorption is too slow. In milder cases or for prophylactic purposes, however, it might be used.

Acknowledgement—My thanks are due to the director of this institute, Professor G. Ljunggren, for the kind interest he has shown in this work.

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Biochemical Pharmacology, 1960, Vol. 5, pp. 231–237. Pergamon Press Ltd., Printed in Great Britain.

FIBROSING PROPERTIES, IN THE RAT, OF COMPOUNDS RELATED TO DICETYL (DI-*n*-HEXADECYL) PHOSPHATE—I*

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(*Received in revised form* 8 *July* 1960)

Abstract—In this preliminary survey, twenty-six compounds, related in one way or another to dicetyl(di-*n*-hexadecyl)phosphate, were compared with this substance as to tissue fibrosing activity by subcutaneous injections in the rat. Only dialkylphosphates (and the single alkylborate used) with a chain length of sixteen or eighteen carbon atoms were active. The only substance with unsaturated alkyl chains, dioleyl(C_{18})phosphate, was inactive. Dicholesteryl-phosphate and phosphite were not active. None of the phosphites (di- or tri-) was active. Dicetylborate produced less fibrosis than the phosphate and this was accompanied by marked eosinophilia and subsequent encapsulated necrosis. Dioctadecyl phosphate was indistinguishable in these experiments from dicetyl phosphate.

USE of sclerosing compounds has long been an accepted method of producing fibroblastic activity in both experimental and clinical medicine. This has been extended from early attempts merely to obliterate varicose veins to a much more diversified use today.

Besides their injection into varicose veins,[1–6] and most certainly less well known, has been the use of materials which initiate fibroblastic activity, in order to obliterate ganglia of the wrists,[7] angiomata,[8] fistulae,[9] hemorrhoids,[10] umbilical herniae,[11] inguinal herniae,[12] idiopathic hydrocele,[13] verrucae plantaris,[14] blood vessels and aneurysms.[15–20] These substances have also been used in situations in which physiology or anatomy is intentionally altered. Among these are the treatment of urinary incontinence,[21] experimental nerve sclerosing,[22] obliteration of the adrenal medulla,[23] and the strengthening or replacement of ligaments and tendons.[24, 25]

Compounds or materials used in these procedures have been as widely diversified as the procedures themselves and have included cellophane or polyethylene,[15–17, 25–31] sodium morrhuate,[20] aspirin,[23] sodium tetradecyl sulfate,[1, 14, 22] and sodium dicetyl (di-*n*-hexadecyl) phosphate.[18, 19, 21, 22]

Interest was stimulated in pure compounds as sclerosing agents when Page[26] produced hypertension in dogs by wrapping their kidneys with cellophane. Poppe

* This study, the first of a series, was aided initially, in part, by a grant from Initiative 171 funds, State of Washington, to one of us (K.A.M.), and currently by a grant from the National Institutes of Health, USPHS, H-2747, to T.L.F.

† To whom correspondence regarding this paper should be sent.

and De Oliveira[17] then studied various types of cellophane, some producing marked fibroblastic activity and some being relatively inert. Several reports followed, giving uses of cellophane or polyethylene in an inert capacity,[25, 32–34] while others appeared describing the fibroblastic activity of these polymers.[16, 27] In an excellent historical review Yeager and Cowley[35] showed that while polyethylene itself was inert, dicetyl phosphate, sometimes used in the processing of these plastic sheets, was a potent fibroblastic stimulant. Cooper *et al.*[28] also arrived at the same conclusion. Since then some attention has been directed toward dicetyl phosphate itself as a fibrosing agent, especially for the treatment of aneurysm.[19a, b]

The present study was initiated in order to examine the fibrosing properties of substances related structurally, in one way or another, to dicetyl phosphate. Other than Reiner's observations[20] regarding some soaps, synthetic detergents, sodium ricinoleate and sodium morrhuate, there seems to be no systematic examination in the literature for structure–activity relationships of any series of fibrosing compounds. We felt it, therefore, of intrinsic interest to find the structural limitations for fibrosing activity in this series. We hoped, also, to gain increased knowledge of the mechanism by which these substances promote formation of fibrous tissue. In addition, there was the possibility of finding a substance more readily applicable and more rapid in its action than dicetyl phosphate.

METHODS

In this initial survey twenty-six compounds (Table 1) related to dicetyl phosphate were tested in comparison with the latter substance. A number of these were synthesized in this laboratory and some are new to the literature. The preparation and chemical properties of the latter will be described in a paper now in preparation in which we will further extend data on the structural requirements for fibrosing activity in this series.

All compounds were given to Wistar rats by subcutaneous injection, using groups of ten animals (male and female) for each substance. The compounds were either dissolved or suspended (5 per cent) in isotonic saline or (10 per cent) in corn oil.*

TABLE 1. SUBSTANCES INVESTIGATED FOR POSSIBLE TISSUE-FIBROSING ACTION, AS COMPARED WITH DICETYL PHOSPHATE

Compound	Formula	Footnote
(1) Didecylphosphate	$(C_{10}H_{21}O)_2\ P(=O)OH$	*
(2) Diundecylphosphate	$(C_{11}H_{23}O)_2\ P(=O)OH$	*

* Suspensions of the long-chain phosphates were made by heating them in corn oil on the water bath, at not over 80 °C, for a short time until they were in solution. They were then allowed to cool and injected at not over 40° as a gel-like mixture.

TABLE 1—*continued.*

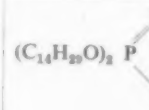
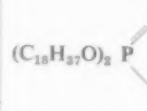
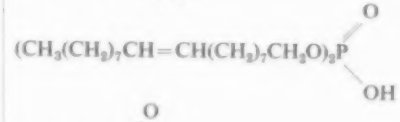
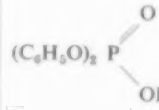
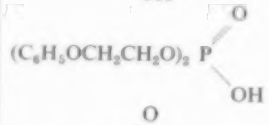
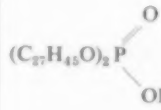
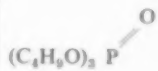
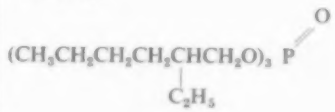
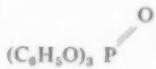
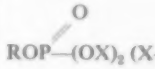
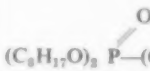
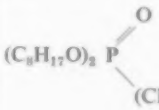
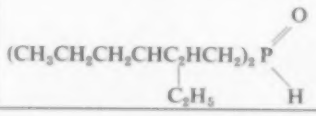
Components	Formula	Footnote
(3) Dimyristyl phosphate		†
(4) Dioctadecyl phosphate		†
(5) Dioleyl phosphate (crude)		*, †, (a)
(6) Diphenyl phosphate		
(7) Diphenoxyethyl phosphate		*
(8) Di (or pyro-) cholesteryl phosphate		§
(9) Tributyl phosphate		‡, (b)
(10) Tri-2-ethylhexyl phosphate		‡, (c)
(11) Triphenyl phosphate		
(12) "Victawcet-14"	 M.W. ~ 500)	‡, (d)
(13) Di*iso*octylphenyl phosphonate		‡, (d)
(14) Di*iso*octylstyryl phosphonate		‡, (d)
(15) Di-2-ethylhexyl phosphite		†, (e)

TABLE 1—*continued.*

Compound	Formula	Footnote
(16) Dicetyl phosphite	$(C_{16}H_{33}O)_2\,P(=O)H$	*
(17) Dicholesteryl phosphite	$(C_{27}H_{45}O)_2\,P(=O)H$	**
(18) Tri (? with di) decyl phosphite	$(C_{10}H_{21}O)_2\,P(=O)H$	*
(19) Tri (? with di) lauryl phosphite	$(C_{12}H_{25}O)_2\,P(=O)H$	*
(20) Tri (? with di) myristyl phosphite	$(C_{14}H_{29}O)_2\,P(=O)H$	*
(21) Tri (? with di) octadecyl phosphite	$(C_{18}H_{37}O)_2\,P(=O)H$	*
(22) Tri-2-ethylhexylphosphite	$(CH_3CH_2CH_2CH_2CH(C_2H_5)CH_2O)_3P$	‡, (e)
(23) Triisooctylphosphite	$(C_8H_{17}O)_3P$	‡, (e), ††
(24) Triphenylphosphite	$(C_6H_5O)_3P$	
(25) Tri (? with di) cetylborate (unstable)	$(C_{16}H_{33}O)_2BOH$	*
(26) Dicetylether	$(C_{16}H_{33})_2O$	‡ (f)

* New compound.
† Made in this laboratory.
‡ These compounds were obtained through the generosity of:
(a) The oleyl alcohol was donated by M. Michel & Co., Inc., N.Y., N.Y.
(b) Commercial Solvents Corporation, N.Y., N.Y.
(c) Carbide and Carbon Chemical Corporation, N.Y., N.Y.
(d) Victor Chemical Works, Chicago 6, Ill.
(e) Virginia-Carolina Chemicals Corporation, Richmond, Va.
(f) Humphrey-Wilkinson, Inc., North Haven, Conn.
§ Awaits characterization: from the reaction of cholesterol and PCl_3 with pyridine in benzene.
** Awaits characterization: from the reaction of cholesterol and PCl_3 with pyridine in benzene.
†† Mixture of octyl isomers.

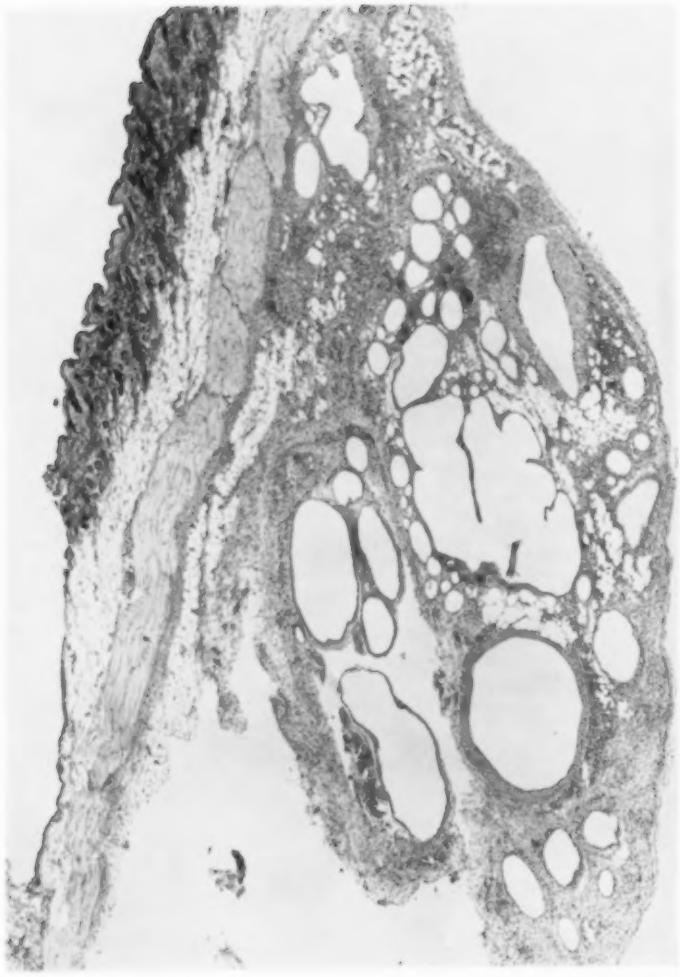


FIG. 1 (a). Control: corn oil injection site at 6 weeks showing persisting sites of corn oil vesicles. This grossly gives the appearance of small vesicular areas bounded by thin avascular walls. × 24.

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FIG. 1 (b). Dicetylphosphate in corn oil: injection site at 6 weeks, showing extensive fibrosis about small central areas of necrosis. Giant cells are appearing in great numbers throughout the densely fibrotic areas. × 24.

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Each compound was injected (0·1 ml) subcutaneously only once in two places (saline and corn oil) on the right abdomen.* Dicetyl phosphate was similarly injected (left abdomen) in all animals for comparison.

Following this, animals were weighed weekly for six weeks to determine toxicity. Microscopic examination was made† of all fibroblastic nodules for each active substance. One-half the surviving animals were sacrificed at 6–8 weeks and the remainder at 6 months following injection.

RESULTS AND DISCUSSION

Compounds showing no fibrosing activity in 6–8 weeks showed no change at 6 months. Active compounds had stimulated the maximum response in 6–8 weeks (or less) and there appeared to be no change at 6 months. We cannot say on the basis of present results that either corn oil or saline injections resulted in greater response to active compounds. The corn oil nodules in general appeared larger; the center of these was usually hollow and contained what seemed to be some of the original corn oil.

Other than dicetylphosphate, only two compounds, dioctadecylphosphate and di (tri?) cetylborate showed appreciable fibrosing activity. The former was approximately equal in activity to dicetylphosphate: the latter was different qualitatively, being accompanied by marked eosinophilia during the first week (see Table 2) and the presence of numerous mast cells.† Central necrosis occurred during the second week and became marked, but was contained by the fibrous capsule. None of the nodules at the sites of dicetyl or dioctadecyl phosphate injection showed appreciable necrosis. See Fig. 1 for representative illustrations.

Compound nos. 11 and 22 (Table 1) seemed to have marked toxicity. All the animals showed signs of debility a day or so after injection and died in a few weeks.

The control groups injected with saline alone had no fibrous nodules. Sites at which corn oil alone was injected developed small encapsulated vesicles with thin avascular walls. None of the latter was thickened or fibrotic.

Table 2 shows the results of an experiment comparing the three active compounds and giving a more detailed picture of the rate of fibrosis. In this experiment three groups of thirty rats each were injected as described above and five of each group were sacrificed at the end of each week.

Before attempting any serious explanation of these findings we are extending our study to include the following compounds: monoalkylphosphates, dialkylphosphates with chain length of more than eighteen carbons, tricetylphosphate, dialkylphosphates with an odd number of carbon atoms (especially diheptadecylphosphate and dipentadecylphosphate) and with branching‡ on the chain, and monocetylphosphite.

Preliminary findings in this projected work, together with the foregoing, indicate that for activity with this type of substance there must be at least one free —OH group, suggesting that enzymes which split esters of phosphoric acid may be involved. The chain must be more than fourteen carbons in length.

* The area to be injected was sponged with alcohol and sterile needles and syringes were used, otherwise no special aseptic precautions were taken and no deaths or recognizable differences were apparent which could be attributed to this aspect of the technique.

† We are indebted to Dr. R. Reiff, formerly of the Department of Pathology, for examining the specimens and slides.

‡ An example of the latter, isolated from wool wax (Australian sheep) is now in our hands.

The only unsaturated alcohol represented thus far (oleyl, C_{18}) may react in a different manner because of the double bond. The fact that the latter was a crude sample (see Table 1) with at least one other component (mono-oleylphosphate) makes it advisable that these results be confirmed before any conclusions are reached about this type.

The apparent abrupt cut-off (C_{16}, active; C_{14}, inactive; C_{15}*, ?) is not understood. If gradual diminution in activity with shortening chain length were observed, we could perhaps assume that greater solubility in the body fluids leads to more rapid assimilation of the smaller molecules, thus removing them from the injection site before action leading to fibrosis takes place. The interesting observation of Theorell, recently discussed,[36] regarding a similar sharp break at C_{15} (fatty acid) in the formation of ternary complexes in the liver–ADH system is provocative, although any relation between these phenomena is not apparent.

TABLE 2. COMPOUNDS SHOWING TISSUE-FIBROSING ACTIVITY COMPARABLE TO THAT OF DICETYLPHOSPHATE (6-WEEK PERIOD)

Week	Dicetylphosphate	Dioctadecylphosphate	Tri (?di) cetylborate
1	Marked fibrosis; little to no eosinophilia	Marked fibrosis; little to no eosinophilia	Moderate fibrosis. Extreme eosinophilia numerous tissue mast cells
2	Fibrosis increasing and penetrating to center of nodule	Fibrosis increasing and penetrating to center of nodule	Central necrosis, with little fibrosis; eosinophilia decreasing
3	Thickly fibrosed, with giant cells appearing in greater numbers	Quite comparable to reaction with dicetyl phosphate	Little fibrosis; necrosis, still some eosinophils but appearing to be a chronic inflammatory response
4	Extensive fibrosis, even into center of large nodules; slight central necrosis	Extensive fibrosis, but perhaps a little less centrally than with dicetyl phosphate	Thinly encapsulated, necrotic mass; eosinophilic response disappearing
5	Extensive fibrosis continuing, although near a maximum	Extensive fibrosis (as with dicetyl phosphate)	Thinly encapsulated necrotic mass; detritus in center of nodules
6	Extensive fibrosis throughout nodules	Extensive fibrosis throughout nodules	No additional fibrosis; necrosis continuing, with thin capsule of inflammatory reaction surrounding central detritus

* We have recently obtained some *n*-pentadecanol through the kindness of the Cancer Chemotherapy Service Center of the U.S. Public Health Service.

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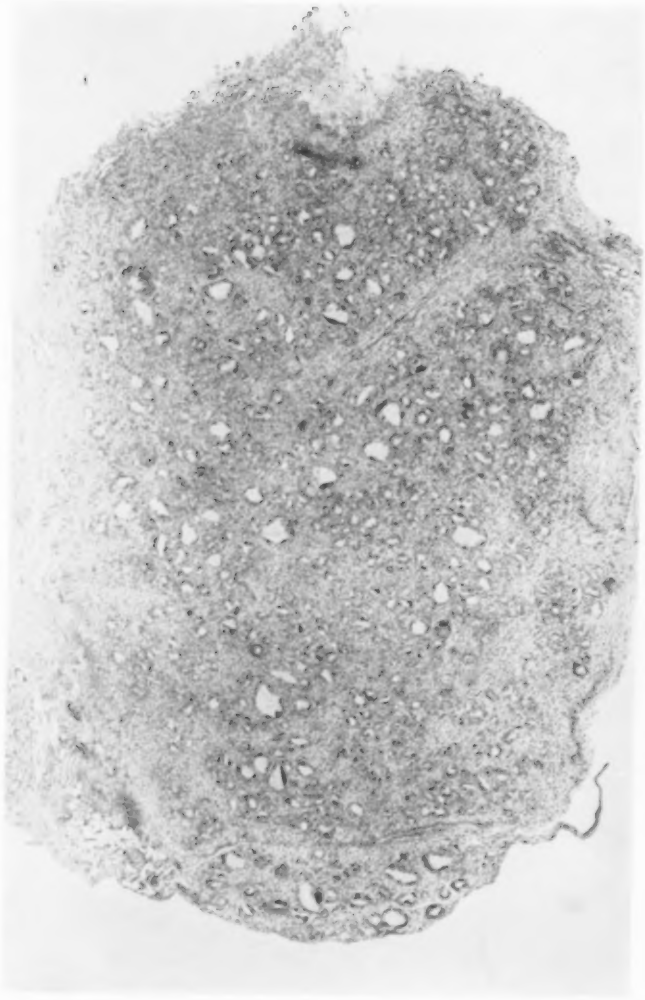

FIG. 1 (c). Dioctadecyl phosphate in normal saline: injection site at 6 weeks, showing extensive fibrosis throughout nodule with only a few limited necrotic areas. Some giant cells are present. × 24.

facing page 236

FIG. 1 (d). Dicetyl borate in normal saline: injection site at 6 weeks, showing small outer margin of fibrosis with a thin wall of inflammatory reaction about central detritus. Many eosinophils and mast cells present. × 88.

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INHIBITION OF THE BIOSYNTHESIS OF PURINES *DE NOVO* BY 6-CHLOROPURINE AND AZASERINE IN SENSITIVE AND NATURALLY RESISTANT ASCITES CELLS*

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(*Received in revised form* 23 *July* 1960)

Abstract—The action of 6-chloropurine and azaserine on the biosynthesis of guanine *de novo* was measured in five ascites-cell neoplasms to determine whether sensitivity to combinations of these agents could be correlated with the blockage of this pathway by the drugs. A positive correlation was obtained with four of these tumors. Azaserine caused essentially complete inhibition of purine biosynthesis, while chloropurine produced a maximum of about 70 per cent retardation of glycine incorporation into polynucleotide guanine in Ehrlich carcinoma and sarcoma 180, neoplasms sensitive to therapy with combinations of chloropurine and azaserine. The 6C3HED and the Mecca lymphosarcomas were relatively resistant to therapy with the drug combination. In the 6C3HED lymphosarcoma, azaserine produced a marked inhibition of purine nucleotide formation. Chloropurine did not affect the incorporation of glycine into polynucleotide guanine; however, it did afford approximately 40 per cent reduction in the rate of isotope utilization for polynucleotide adenine in this tumor. In the Mecca lymphosarcoma, both drugs were unable to affect guanine formation *de novo* to the same extent found in the sensitive neoplasms. However, with hepatoma 134, both azaserine and chloropurine caused a reduction in the rate of guanine formation comparable to the inhibition obtained in sensitive tumors, although this neoplasm is not sensitive to the drug combination. The possible interpretations of these findings have been discussed.

INTRODUCTION

6-CHLOROPURINE has been found to potentiate the tumor inhibition produced by the glutamine analogs, azaserine (O-diazoacetyl-L-serine) or DON (6-diazo-5-oxo-L-norleucine), in a number of experimental tumor lines.[1, 2] Studies on the mode of action of azaserine in mammalian systems have indicated that this agent produced intensive inhibition of the biosynthesis of purines *de novo*.[3–10] Since earlier work[11, 12] showed that 6-chloropurine retarded the incorporation of radioactive glycine into polynucleotide guanine without altering the rate of incorporation of glycine into adenine nucleotides, the possibility therefore existed that the tumor-inhibitory properties of combinations of chloropurine and azaserine were due to the formation of two mutually dependent blocks which limited the synthesis of guanine nucleotides. Simultaneous

* The following abbreviations are used: IMP for inosine 5′-phosphate, NA for nucleic acid, and AS for acid-soluble.

administration of these two drugs was essential for carcinostasis.[2] This finding was consistent with the theory that these agents established complementary metabolic blocks on pathways leading to purine nucleotide biosynthesis. To determine whether these biochemical events were related to the tumor-inhibitory properties of the drug combination, the effects of azaserine and chloropurine on the formation of purines *de novo* were compared in susceptible and naturally resistant ascites tumors.

MATERIALS AND METHODS

Five mouse ascites-cell tumors were employed in these experiments: sarcoma 180 and Ehrlich carcinoma in female Ha/ICR Swiss mice (A. R. Schmidt Co., Inc., Madison, Wis.), 6C3HED lymphosarcoma and hepatoma 134 in female C3H mice (Texas Inbred Mice Co., Houston, Tex.), and Mecca lymphosarcoma in female AKR mice (Texas Inbred Mice Co.). Animals were inoculated intraperitoneally six

TABLE 1. THE BIOSYNTHESIS OF PURINES *de novo* IN SARCOMA 180 ASCITES CELLS TREATED WITH AZASERINE

Hours after azaserine dose	Counts/min per μmole $\times 10^{-2}$		
	NA guanine	NA adenine	AS adenine
0	4·0 ± 0·4*	3·3 ± 0·5	58·3 ± 5·0
1	0·1 ± 0·05	0·07 ± 0·1	0·1 ± 0·03
3	0·1 ± 0·04	0·08 ± 0·03	0·5 ± 0·1
6	0·2 ± 0·1	0·1 ± 0·01	4·0 ± 1·4
12	0·4 ± 0·1	0·3 ± 0·1	7·3 ± 1·2
24	0·7 ± 0·04	0·4 ± 0·05	7·6 ± 1·1

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with 0·2 mg azaserine per kg. At the designated time, 100 μg glycine-2-^{14}C (10^4 counts/min per μg) per mouse were injected and were allowed to be incorporated for 1 hr. The zero time point represents the results obtained with control mice that received an injection of isotonic saline 1 hr before radioactive glycine.

days before use with 0·1 ml of ascites cell suspension prepared by centrifuging the cells for 2 min in a clinical centrifuge (1600 *g*) and suspending the packed cells in 10 vols. of isotonic saline.

Drugs were dissolved in isotonic saline and injected intraperitoneally: azaserine at a dose of 0·2 mg/kg and chloropurine (Nutritional Biochemicals Corp., Cleveland, Ohio) at a level of 40 mg/kg. Control animals received a comparable volume of isotonic saline in place of the drug. At various times after this dose, each mouse received an intraperitoneal injection of 100 μg of glycine-2-^{14}C (Tracerlab, Inc., Waltham, Mass.) (10^4 counts/min per μg). One hour later, the cells were harvested, and the nucleic acid purines and acid-soluble adenine were isolated and analyzed as described by LePage.[13] Radioactivity was measured with a Nuclear-Chicago Model D47 windowless gas-flow counter. Each experimental point represents the average of values obtained from the separate analyses of ascites cells from eight to twelve mice.

RESULTS

The effect of azaserine on the biosynthesis of purines *de novo* in sarcoma 180, a neoplasm in which chloropurine enhances the carcinostatic effect of azaserine, is shown in Table 1. Glycine-2-^{14}C incorporation into polynucleotide purines was used as the measure of purine synthesis. Injection of radioactive glycine 1 hr after the administration of azaserine resulted in essentially complete inhibition of glycine incorporation into nucleic acid purines and acid-soluble adenine. More than 80 per cent inhibition of this pathway remained 24 hr after the azaserine dose. In this neoplasm, chloropurine administration 1 hr prior to glycine-2-^{14}C resulted in 75 per cent inhibition of isotope incorporation into nucleic acid guanine, with inhibition persisting up to 6 hr after the chloropurine dose.[12]

The effects of these two agents on purine formation were studied using the Ehrlich carcinoma, a tumor which resembled sarcoma 180 in its sensitivity to combinations of these drugs. The results obtained are presented in Table 2. The findings parallelled

TABLE 2. THE BIOSYNTHESIS OF PURINES *de novo* IN EHRLICH CARCINOMA ASCITES CELLS TREATED WITH AZASERINE OR CHLOROPURINE

Drug	Hours after drug dose	Counts/min per μmole × 10^{-2}		
		NA guanine	NA adenine	AS adenine
Azaserine	0	7·3 ± 1·1*	5·3 ± 2·1	105·6 ± 10·9
	1	0·2 ± 0·1	0·04 ± 0·01	0·2 ± 0·07
	12	1·5 ± 0·1	1·0 ± 0·2	26·6 ± 2·7
	24	1·6 ± 0·3	0·9 ± 0·2	21·0 ± 3·3
Chloropurine	0	7·9 ± 1·2	5·2 ± 0·5	113·8 ± 7·3
	5 (min)	3·3 ± 0·3	6·5 ± 0·8	125·5 ± 16·5
	1	2·9 ± 0·4	4·8 ± 0·6	102·7 ± 12·5
	6	6·0 ± 0·6	6·4 ± 0·5	126·4 ± 4·8

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with either 0·2 mg azaserine per kg or 40 mg chloropurine per kg. At the designated time, 100 μg glycine-2-^{14}C (10^4 counts/min per μg) per mouse were injected and were allowed to be incorporated for 1 hr. The zero time point represents the results obtained with control mice that received an injection of isotonic saline 1 hr before radioactive glycine.

those obtained with sarcoma 180; azaserine produced marked, prolonged inhibition of purine synthesis, and chloropurine produced a maximum retardation of glycine incorporation into nucleic acid guanine of 63 per cent. No inhibition of the formation of polynucleotide adenine occurred in chloropurine-treated cells.

Table 3 presents the results of the experiments using 6C3HED ascites cells. Chloropurine was unable to enhance the tumor-inhibitory properties of azaserine in this lymphosarcoma. Azaserine treatment produced essentially complete inhibition of glycine incorporation into cellular purines, with marked inhibition still present up to 12 hr after the drug dose. In contrast to results obtained with the other neoplasms, chloropurine did not prevent the synthesis of polynucleotide guanine *de novo*, but did produce approximately 40 per cent retardation of glycine utilization for nucleic acid

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adenine when the purine analog was administered between 1 and 12 hr before glycine-2-^{14}C.

Table 4 shows the data obtained using another tumor insensitive to the drug combination, the Mecca lymphosarcoma. Administration of glycine-2-^{14}C 1 hr after a dose of azaserine or chloropurine resulted in considerably less inhibition of isotope incorporation than was found with the sensitive neoplasms.

TABLE 3. THE BIOSYNTHESIS OF PURINES *de novo* IN 6C3HED LYMPHOSARCOMA ASCITES CELLS TREATED WITH AZASERINE OR CHLOROPURINE

Drug	Hours after drug dose	Counts/min per μmole $\times 10^{-2}$		
		NA guanine	NA adenine	AS adenine
Azaserine	0	8·7±0·7*	18·5±1·7	242·2±17·9
	1	0·4±0·1	0·2±0·1	4·9± 2·3
	3	0·7±0·2	0·3±0·06	5·4± 0·6
	6	1·5±0·3	1·5±0·8	19·6± 4·9
	12	3·0±1·2	6·5±2·5	38·1±11·9
Chloropurine	0	10·0±1·2	17·2±2·8	227·0±27·5
	5 (min)	9·9±1·2	19·0±4·0	269·8±20·4
	1	8·3±1·4	9·0±1·2	182·7± 7·7
	3	7·7±0·7	10·3±1·7	202·8±22·9
	6	9·8±1·8	10·1±1·2	170·6±26·4
	12	8·2±1·3	9·1±0·5	163·2± 2·4

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with either 0·2 mg azaserine per kg or 40 mg chloropurine per kg. At the designated time, 100 μg glycine-2-^{14}C (10^4 counts/min per μg) per mouse were injected and were allowed to be incorporated for 1 hr. The zero time point represents the results obtained with control mice that received an injection of isotonic saline 1 hr before radioactive glycine.

TABLE 4. THE BIOSYNTHESIS OF PURINES *de novo* IN MECCA LYMPHOSARCOMA ASCITES CELLS TREATED WITH AZASERINE OR CHLOROPURINE

Drug	Counts/min per μmole $\times 10^{-2}$		
	NA guanine	NA adenine	AS adenine
Control	1·6±0·1*	1·6±0·2	28·7±3·0
Azaserine	0·9±0·1	0·6±0·09	2·8±0·4
Chloropurine	1·2±0·5	1·6±0·4	27·2±5·2

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with either 0·2 mg azaserine per kg or 40 mg chloropurine per kg. One hour later, 100 μg glycine-2-^{14}C (10^4 counts/min per μg) per mouse were injected and were allowed to be incorporated for 1 hr. Control mice received an injection of isotonic saline 1 hr before radioactive glycine.

The results of experiments carried out with hepatoma 134 are given in Table 5. This neoplasm is susceptible to azaserine treatments; however, addition of chloropurine to this regimen did not elicit a further therapeutic response. Both azaserine

and chloropurine produced metabolic blocks of a magnitude and a duration comparable to those found using sarcoma 180 or Ehrlich carcinoma.

Chloropurine was without effect on the incorporation of glycine-2-^{14}C into residual protein in all of the neoplasms studied.

TABLE 5. THE BIOSYNTHESIS OF PURINES *de novo* IN HEPATOMA 134 ASCITES CELLS TREATED WITH AZASERINE OR CHLOROPURINE

Drug	Hours after drug dose	Counts/min per μmole $\times 10^{-3}$		
		NA guanine	NA adenine	AS adenine
Azaserine	0	4·2±1·0*	4·9±1·0	134·8±16·9
	1	0·4±0·06	0·5±0·1	16·2± 4·2
	12	0·5±0·1	0·8±0·1	30·3± 4·4
	24	1·3±0·4	1·7±0·4	45·4±10·4
Chloropurine	0	2·0±0·1	4·3±0·3	110·6± 8·8
	5 (min)	1·2±0·1	5·2±0·3	129·4± 9·3
	1	0·7±0·07	3·7±0·3	95·9± 7·3
	6	1·3±0·1	4·5±0·3	128·2± 8·8

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with either 0·2 mg azaserine per kg or 40 mg chloropurine per kg. At the designated time, 100 μg glycine-2-^{14}C (10^8 counts/min per μg) per mouse were injected and were allowed to be incorporated for 1 hr. The zero time point represents the results obtained with control mice that received an injection of isotonic saline 1 hr before radioactive glycine.

DISCUSSION

Measurements of the rates of incorporation of various isotopic precursors into polynucleotide purines after chloropurine treatment have indicated that this analog produced a metabolic block on the route to nucleic acid guanine formation *de novo*. The conversion of 5-amino-4-imidazolecarboxamide ribonucleotide to inosine 5′-phosphate (IMP) or the conversion of IMP to guanosine 5′-phosphate was considered to be the probable site of the sensitive enzyme.[11, 12] The major site of action of azaserine on this pathway is the conversion of α-N-formylglycinamide ribonucleotide to α-N-formylglycinamidine ribonucleotide.[4, 7–10]

Measurements of the magnitude and duration of the blocks produced by chloropurine and azaserine were made in a spectrum of ascitic neoplasms exhibiting differences in susceptibility to these agents in order to determine whether these blocks were associated with the carcinostatic properties of the drug combination. The results obtained are presented in Table 6. Data pertaining to inhibition of tumor growth are summarized from earlier experiments.[2] Azaserine prolonged the survival time of mice bearing Ehrlich carcinoma or sarcoma 180, while chloropurine was inactive. Combination of these agents in the therapy of these malignancies resulted in enhanced antitumor properties. In these two tumors, both azaserine and chloropurine produced metabolic blocks on the pathway to purine formation *de novo*. The 6C3HED lymphosarcoma showed some sensitivity to azaserine. This was in accord with the biochemical inhibition of purine biosynthesis by this antibiotic. The inability of

chloropurine to enhance the action of azaserine correlated with the lack of inhibition of glycine-^{14}C incorporation into nucleic acid guanine by the purine analog. This lack of inhibition did not appear to be due to cellular permeability, for chloropurine caused a retardation of radioactive glycine utilization for polynucleotide adenine. The Mecca lymphosarcoma was relatively resistant to drug therapy. This appeared to be due to the inability of both drugs to produce blocks of a magnitude found in the sensitive neoplasms.

Hepatoma 134 proved to be an exception. The growth of these cells was inhibited by azaserine; addition of chloropurine did not elicit further therapeutic response. However, biochemically each agent accomplished the metabolic blocks measured in a magnitude comparable to those found in the sensitive Ehrlich carcinoma and sarcoma 180. These results suggest that either the chloropurine and azaserine blocks which

TABLE 6. SUMMARY OF EFFECTS OF AZASERINE AND CHLOROPURINE ON SENSITIVE AND NATURALLY RESISTANT ASCITES TUMORS

Neoplasm	Inhibition of tumor growth*			Biochemical inhibition†	
	Azaserine	Chloropurine	Azaserine + chloropurine	Synthesis of purines *de novo* (azaserine)	Glycine-2-^{14}C into NA guanine (chloropurine)
Sarcoma 180	+	−	++	+	+
Ehrlich	+	−	++	+	+
6C3HED	+	−	+	+	−
Mecca	−	−	−	±	±
Hepatoma 134	+	−	+	+	+

* Degree of inhibition of ascites tumor growth as measured by prolongation of survival time: − indicates no effect; + indicates significant prolongation of survival; ++ indicates enhanced anti-tumor properties. Inhibition of 6C3HED was obtained using a measurement of the total packed cell volume.

† Degree of biochemical inhibition: − indicates no inhibition; + indicates a minimum of 90 per cent inhibition with azaserine and a minimum of 60 per cent inhibition with chloropurine.

were measured were unrelated to the growth inhibition by the drug combination, or that they were only part of the reason for the enhanced antineoplastic properties. However, it was also conceivable that the lack of correlation exhibited by hepatoma 134 was due to differences in the relative importance of alternate metabolic routes. This possibility was investigated in the following paper[14], using a number of preformed purines.

Acknowledgements—The authors wish to thank Mrs. Eunice Brown for excellent technical assistance. Azaserine was generously supplied by Dr. John R. Dice, Research Division, Parke, Davis and Company.

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Biochemical Pharmacology, 1960, Vol. 5, pp. 245–250. Pergamon Press Ltd., Printed in Great Britain.

UTILIZATION OF PREFORMED PURINES BY SARCOMA 180 OR HEPATOMA 134 CELLS TREATED WITH 6-CHLOROPURINE OR A COMBINATION OF 6-CHLOROPURINE AND AZASERINE*

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(*Received in revised form* 27 *July* 1960)

Abstract—The metabolism of preformed purines was compared in sarcoma 180 and hepatoma 134 ascites tumor cells exposed to either 6-chloropurine or a combination of chloropurine and azaserine. Marked alterations in purine utilization occurred in the drug-sensitive cells (sarcoma 180) treated with chloropurine. These were an increased incorporation of adenine and guanine into polynucleotide guanine, an increased conversion of guanine to acid-soluble adenine, and an increased conversion of hypoxanthine to both polynucleotide and acid-soluble adenine. Preformed purine metabolism of hepatoma 134, a neoplasm in which chloropurine did not enhance the growth-retarding properties of azaserine, proved to be relatively refractive to chloropurine. An increased conversion of preformed guanine to acid-soluble adenine occurred as a consequence of chloropurine treatment. Exposure of sarcoma 180 to a combination of chloropurine and azaserine increased the rate of conversion of preformed purines to adenine nucleotides over that found with chloropurine alone. Hepatoma 134 also responded to the drug combination by an increase in the rate of preformed purine conversion to adenine nucleotides. These reactions were interpreted as manifestations of compensatory responses to the stress of metabolic blocks on the pathway of purine synthesis *de novo*.

INTRODUCTION

IN THE previous paper[1] the tumor-inhibitory properties of combinations of 6-chloropurine and azaserine were found to correlate with the blockage of guanine nucleotide biosynthesis *de novo* by each agent in four ascites cell neoplasms. Hepatoma 134 was an exception, for both chloropurine and azaserine produced a decreased rate of guanine formation comparable to that obtained with sensitive tumors, although this neoplasm was not sensitive to the drug combination. Since it was conceivable that the growth-retarding properties of this dual block were minimized in hepatoma 134 by differences in the relative importance of alternate metabolic routes, the ability of this neoplasm to utilize preformed purines was compared with sensitive sarcoma 180 cells during exposure to chloropurine or a combination of chloropurine and azaserine. A preliminary report of this work has been presented.[2]

MATERIALS AND METHODS

Sarcoma 180 ascites cells were maintained in female Ha/ICR Swiss mice (The A. R. Schmidt Co., Madison, Wis.), and hepatoma 134 ascites cells were maintained

* The following abbreviations are used: NA for nucleic acid and AS for acid-soluble.

in female C3H mice (Texas Inbred Mice Co., Houston, Texas). Each tumor was transplanted 6 days before use into mice of the C3H strain. Cells were withdrawn from donor animals, centrifuged for 2 min in a clinical centrifuge (1600 *g*), suspended in 10 vols. of isotonic saline and 0·1 ml of the suspension was injected into each mouse.

Drugs were dissolved in isotonic saline and injected intraperitoneally: azaserine at a dose of 0·2 mg/kg and chloropurine (Nutritional Biochemicals Corp., Cleveland, Ohio) at a level of 40 mg/kg. At various times after the injection of the drug or drugs, each mouse received an intraperitoneal injection of either 50 μg of guanine-8-^{14}C (California Corp. for Biochemical Research, Los Angeles, Calif.) (12×10^3 counts/min per μg), 50 μg of adenine-8-^{14}C (California Corp. for Biochemical Research) (14×10^4 counts/min per μg), or 50 μg of hypoxanthine-8-^{14}C (Nuclear-Chicago Corp., Chicago, Ill.) ($9{\cdot}4 \times 10^3$ counts/min per μg). Guanine-8-^{14}C was allowed to incorporate for 20 min, while adenine-8-^{14}C and hypoxanthine-8-^{14}C were allowed 1 hr. At the end of the incorporation period, the cells were harvested, and nucleic acid purines and acid-soluble adenine were isolated and analyzed as described previously.[1] Each experimental point represents the average of values obtained from separate analyses of ascites cells from four to eight mice.

RESULTS

In earlier reports,[2, 3] inhibition of the formation of guanine *de novo* by chloropurine in sarcoma 180 ascites cells was shown to be accompanied by increased utilization of certain preformed purines. This apparent compensatory mechanism was compared in cells sensitive (sarcoma 180) and resistant (hepatoma 134) to combinations of azaserine and chloropurine by measuring the effect of chloropurine on the rate of guanine-8-^{14}C utilization for purine formation (Table 1). Untreated cells of both tumors appeared to

TABLE 1. EFFECT OF 6-CHLOROPURINE ON GUANINE-8-^{14}C INCORPORATION INTO PURINES OF SARCOMA 180 AND HEPATOMA 134 CELLS

Neoplasm	Hours after chloropurine dose	Counts/min per μmole $\times 10^{-5}$		
		NA guanine	NA adenine	AS adenine
Sarcoma 180	0	20·2 ± 1·7*	1·4 ± 0·2	48·0 ± 2·8
	1	23·5 ± 3·2	1·3 ± 0·3	67·1 ± 9·8
	3	35·0 ± 4·9	1·9 ± 0·4	154·8 ± 32·5
Hepatoma 134	0	24·2 ± 1·3	1·1 ± 0·1	92·3 ± 10·7
	1	23·6 ± 2·0	1·1 ± 0·05	215·9 ± 45·9
	3	20·3 ± 0·7	0·9 ± 0·1	153·9 ± 23·8

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with 40 mg chloropurine per kg. At the designated time, 50 μg guanine-8-^{14}C (12×10^3 counts/min per μg) per mouse were injected and were allowed to be incorporated for 20 min. The zero time points represent the results obtained with control mice that received an injection of isotonic saline 1 hr before radioactive guanine.

utilize guanine for polynucleotide formation at similar rates. Following chloropurine treatment, an increased incorporation of isotope into polynucleotide guanine and acid-soluble adenine was obtained in sarcoma 180 cells, while only the conversion of radioactive guanine to acid-soluble adenine was increased in treated hepatoma 134.

The changes in adenine metabolism induced by chloropurine were estimated in these two neoplasms by determining the effect of the purine analog on adenine-8-^{14}C uptake (Table 2). Both tumors utilized preformed adenine. Chloropurine administration caused a stimulation of adenine conversion to guanine nucleotides in sarcoma 180 tumor-bearing mice. No significant alterations in adenine incorporation into purines occurred in drug-treated hepatoma 134 cells.

TABLE 2. EFFECT OF 6-CHLOROPURINE ON ADENINE-8-^{14}C INCORPORATION INTO PURINES OF SARCOMA 180 AND HEPATOMA 134 CELLS

Neoplasm	Hours after chloropurine dose	Counts/min per μmole $\times 10^{-2}$		
		NA guanine	NA adenine	AS adenine
Sarcoma 180	0	4·5±0·3*	78·3±6·3	1290± 54
	1	8·1±1·8	67·9±9·2	1215±116
	3	8·4±1·9	58·9±9·3	1116± 72
Hepatoma 134	0	3·7±0·3	49·3±4·3	759± 72
	1	3·1±0·3	43·5±3·1	713± 28
	3	3·6±0·5	39·2±4·7	733± 66

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with 40 mg chloropurine per kg. At the designated time, 50 μg adenine-8-^{14}C (14×10^4 counts/min per μg) per mouse were injected and were allowed to be incorporated for 1 hr. The zero time points represent the results obtained with control mice that received an injection of isotonic saline 1 hr before radioactive adenine.

TABLE 3. EFFECT OF 6-CHLOROPURINE ON HYPOXANTHINE-8-^{14}C INCORPORATION INTO PURINES OF SARCOMA 180 AND HEPATOMA 134 CELLS

Neoplasm	Hours after chloropurine dose	Counts/min per μmole $\times 10^{-2}$		
		NA guanine	NA adenine	AS adenine
Sarcoma 180	0	12·0±1·1*	15·2±1·0	264·2±13·4
	1	12·6±0·9	29·5±0·8	446·5±13·2
	3	8·8±1·5	18·6±1·4	335·1±15·3
	6	12·3±2·2	18·2±1·4	311·2±15·1
Hepatoma 134	0	4·4±0·6	13·6±1·3	210·4±14·8
	1	2·8±0·2	14·1±0·5	235·5± 6·7
	3	3·2±0·3	12·8±0·7	211·6± 9·4
	6	5·3±0·4	14·6±1·3	189·2± 8·4

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with 40 mg chloropurine per kg. At the designated time, 50 μg hypoxanthine-8-^{14}C ($9{\cdot}4 \times 10^3$ counts/min per μg) per mouse were injected and were allowed to be incorporated for 1 hr. The zero time points represent the results obtained with control mice that received an injection of isotonic saline 1 hr before radioactive hypoxanthine.

Table 3 shows the effects of the drug on hypoxanthine-8-^{14}C incorporation into purines of the two tumors. Hypoxanthine appeared to be utilized for polynucleotide guanine formation at a greater rate in sarcoma 180 cells, while the conversion of this metabolite to adenine compounds was similar in the two cell lines. In sarcoma 180,

chloropurine treatment produced a stimulation of hypoxanthine conversion to adenine compounds while, in contrast, no such effect was obtained with the hepatoma.

In order to estimate the effects of a more intense blockage of the synthesis of purines *de novo*, the action of simultaneous administration of azaserine and chloropurine on preformed purine metabolism was compared in the two neoplasms. The

TABLE 4. EFFECT OF SIMULTANEOUS ADMINISTRATION OF 6-CHLOROPURINE AND AZASERINE ON GUANINE-8-^{14}C INCORPORATION INTO PURINES OF SARCOMA 180 AND HEPATOMA 134 CELLS

Neoplasm	Hours after drugs	Counts/min per μmole $\times 10^{-2}$		
		NA guanine	NA adenine	AS adenine
Sarcoma 180	0	21·8 ± 5·4*	1·1 ± 0·2	78·9 ± 7·3
	1	27·7 ± 3·0	1·5 ± 0·2	145·2 ± 16·0
	3	39·8 ± 7·1	1·6 ± 0·2	183·1 ± 19·7
Hepatoma 134	0	19·3 ± 2·7	0·9 ± 0·3	93·6 ± 10·0
	1	34·5 ± 10·5	1·3 ± 0·4	149·0 ± 27·1
	3	20·2 ± 3·1	0·8 ± 0·04	253·2 ± 44·2

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with 40 mg chloropurine per kg and 0·2 mg azaserine per kg. At the designated time, 50 μg guanine-8-^{14}C (12×10^3 counts/min per μg) per mouse were injected and were allowed to be incorporated for 20 min. The zero time points represent the results obtained with control mice that received an injection of isotonic saline 1 hr before radioactive guanine.

TABLE 5. EFFECT OF SIMULTANEOUS ADMINISTRATION OF 6-CHLOROPURINE AND AZASERINE ON ADENINE-8-^{14}C INCORPORATION INTO PURINES OF SARCOMA 180 AND HEPATOMA 134 CELLS

Neoplasm	Hours after drugs	Counts/min per μmole $\times 10^{-2}$		
		NA guanine	NA adenine	AS adenine
Sarcoma 180	0	4·7 ± 0·4*	58·8 ± 1·5	970 ± 36
	1	5·6 ± 0·4	75·2 ± 5·6	1303 ± 78
	3	8·0 ± 0·8	70·6 ± 3·5	1535 ± 102
Hepatoma 134	0	4·4 ± 1·1	48·0 ± 4·3	800 ± 100
	1	3·6 ± 0·1	58·9 ± 3·1	1066 ± 77
	3	4·6 ± 0·9	64·8 ± 10·4	1353 ± 195

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with 40 mg chloropurine per kg and 0·2 mg azaserine per kg. At the designated time, 50 μg adenine-8-^{14}C (14×10^4 counts/min per μg) per mouse were injected and were allowed to be incorporated for 1 hr. The zero time points represent the results obtained with control mice that received an injection of isotonic saline 1 hr before radioactive adenine.

results of experiments measuring the effects of this drug combination on guanine-8-^{14}C utilization are presented in Table 4. The results obtained with sarcoma 180 cells were similar to those obtained with the purine antagonist alone. Treatment of hepatoma 134 with the drug combination resulted in an increased utilization of guanine for acid-soluble adenine formation. Although this conversion appeared to be less than that found with chloropurine treatment at 1 hr, it was greater at 3 hr.

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Table 5 shows the effects of the two drugs on adenine-8-^{14}C uptake. In the sarcoma, stimulation of incorporation into both adenine and guanine occurred following exposure to drugs; however, the increased incorporation into polynucleotide guanine at 1 hr was less than that found with chloropurine alone. In hepatoma 134, azaserine and chloropurine appeared to stimulate adenine-^{14}C utilization for both nucleic acid and acid-soluble adenine. No increase in the conversion to guanine nucleotides was noted.

The data in Table 6 show that the conversion of hypoxanthine-8-^{14}C to adenine and guanine in sarcoma 180 treated with the two antimetabolites was similar to that

TABLE 6. EFFECT OF SIMULTANEOUS ADMINISTRATION OF 6-CHLOROPURINE AND AZASERINE ON HYPOXANTHINE-8-^{14}C INCORPORATION INTO PURINES OF SARCOMA 180 AND HEPATOMA 134 CELLS

Neoplasm	Hours after drugs	Counts/min per μmole $\times 10^{-2}$		
		NA guanine	NA adenine	AS adenine
Sarcoma 180	0	17·9 ± 2·3*	18·8 ± 1·7	322·9 ± 13·9
	1	16·8 ± 1·5	33·5 ± 1·6	707·2 ± 65·5
	3	13·2 ± 0·7	27·3 ± 0·4	605·7 ± 118
Hepatoma 134	0	5·6 ± 0·5	11·6 ± 1·1	220·8 ± 18·4
	1	3·3 ± 0·4	22·8 ± 0·5	429·8 ± 5·1
	3	4·5 ± 0·2	27·3 ± 3·1	432·0 ± 48·4

* Standard deviation of the mean.
Tumor-bearing mice were each injected intraperitoneally with 40 mg chloropurine per kg and 0·2 mg azaserine per kg. At the designated time, 50 μg hypoxanthine-8-^{14}C ($9{\cdot}4 \times 10^3$ counts/min per μg) per mouse were injected and were allowed to be incorporated for 1 hr. The zero time points represent the results obtained with control mice that received an injection of isotonic saline 1 hr before radioactive hypoxanthine.

obtained with chloropurine treatment, except that incorporation into acid-soluble adenine was greater than that produced by the purine analog alone. Treatment of hepatoma cells with azaserine and chloropurine caused a marked stimulation in the conversion of radioactive hypoxanthine to adenine compounds. The effect on the conversion to guanine was essentially the same as that produced by chloropurine.

DISCUSSION

Differences in the rates of utilization of preformed purines were found in comparisons of sarcoma 180 and hepatoma 134 ascites cells exposed to chloropurine or a combination of chloropurine and azaserine. Treatment of the drug-sensitive cells (sarcoma 180) with chloropurine caused marked alterations in preformed purine metabolism. These were suggestive of cellular compensatory reactions in response to the duress produced by blockage of a relatively important metabolic reaction(s). The changes included an increased incorporation of adenine and guanine into polynucleotide guanine, an increased conversion of hypoxanthine to adenine compounds, and an increased conversion of preformed guanine to acid-soluble adenine. It is of interest to note that the increased rate of guanine-8-^{14}C incorporation into sarcoma 180 polynucleotide guanine, which was found following chloropurine treatment in this study, did not

occur as soon after exposure to the drug as was previously reported.[3] This difference may be a manifestation of tumor growth in different mouse strains, for earlier experiments[3] were carried out using the mouse strain in which the tumor is normally maintained (Ha/ICR Swiss), while the present experiments were carried out in C3H mice.

In contrast to the sarcoma, preformed purine metabolism of hepatoma 134 cells was relatively unaffected by chloropurine treatment; however, exposure to chloropurine did increase the conversion of guanine-8-^{14}C to acid-soluble adenine. The relative refractiveness of preformed purine metabolism of the hepatoma to chloropurine would appear to correlate with the inability of this agent to potentiate the growth-inhibitory properties of azaserine in this neoplasm.

Since chloropurine caused an inhibition of guanine synthesis *de novo* without concurrent inhibition of adenine formation, a combined treatment of azaserine and chloropurine should provide a more complete block of purine biosynthesis *de novo*. This multiple stress would be expected to create a need for adenine, in addition to the apparent increased cellular requirement for guanine observed in chloropurine-treated cells. Measurement of the utilization of preformed purines after exposure to this drug combination suggested that these requirements were indeed created. Sarcoma 180 and hepatoma 134 ascites cells treated with the combination of drugs showed, in general, a greater increase in the rate of conversion of isotopic purines to adenine nucleotides than that found with chloropurine alone. Differences in the response of the two neoplasms to the drug combination appeared to reside in the utilization of precursors for nucleic acid guanine biosynthesis. An increase occurred in the incorporation of adenine and guanine into polynucleotide guanine of sarcoma 180 cells treated with the drugs, while these agents had no significant effect on these reactions in the hepatoma.

Interpretation of the data obtained with chloropurine as compensatory reactions to the imposed stress correlated with the degree of sensitivity of the two neoplasms to therapy with the drug combination. The findings, however, do not appear to explain the inability of chloropurine to enhance the growth-retarding properties of azaserine against hepatoma 134. Indeed, it is probable that inhibition of additional metabolic pathways, hitherto undiscovered, is required for drug enhancement. In the area of purine catabolism, purified milk xanthine oxidase and purified uricase have been indicated as chloropurine-sensitive enzymes by Duggan and Titus.[4] Inhibition of these enzymes *in vivo* might be expected to provide an increased rate of preformed purine conversion to polynucleotides by conservation of substrate molecules. Such an effect may be in part responsible for the results obtained with chloropurine-treated sarcoma 180 cells.

Acknowledgements—We are grateful to Mrs. Eunice Brown for valuable technical assistance. A gift of azaserine was made by Dr. John R. Dice, Research Division, Parke, Davis and Company.

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EFFECTS OF SINGLE OR REPEATED TREATMENT WITH SEVERAL ANTICHOLESTEROLEMIC COMPOUNDS ON BILIARY EXCRETION OF CHOLESTEROL

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(*Received* 22 *August* 1960)

Abstract—The effects of single or repeated treatments with nicotinic acid, 3-pyridineacetic acid, inositol hexanicotinate, Na-phenylbutyrate, betaine phenylbutyrate, 1 : 4-dicaffeylquinic acid and sodium dehydrocholate on biliary excretion of cholesterol were studied in the rat. After a single intravenous administration, among the drugs experimented only 1 : 4-dicaffeylquinic acid at the highests doses examined and phenylbutyrates, these at the lowest doses, increased the biliary excretion of cholesterol. After repeated administrations by the intraperitoneal route none of the compounds examined succeeded in modifying in a statistically significant manner the biliary excretion of cholesterol.

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The cholesterol (CHOL) in the bile is nowadays considered to be a quota of the sterol synthetized by the hepatic parenchyma. In fact most authors consider the biliary and ematic rates of CHOL to be absolutely independent. It is usually maintained that the biliary concentrations of the sterol cannot be modified in any appreciable measure by variations of the content of sterol in the diet, by eventual administration of such by means of injections, or by chronic hypercholesterolemic conditions.

According to Friedman and Byers[1] "the biliary cholesterol appears to represent a rather fixed function of that cholesterol synthetized or discharged from the hepatic cell itself".

On the other hand, research carried out with radio-active isotopes seems to permit the assumption that at least a part of the biliary CHOL is derived from the fraction of the free plasmatic CHOL. In 1943 Bloch *et al.*[2] affirmed that "after injection of deuterium CHOL into a dog, the activity of biliary cholesterol was considerably less than that of hepatic cholesterol, but similar to that of blood, suggesting the probability that bile cholesterol represented a moiety of plasma cholesterol".

Very recently Rosenfeld and Hellman[3] administered CHOL-^{14}C orally to human subjects with complete biliary fistula. They found CHOL-^{14}C in the bile after too short a time to exclude an effective passage of the sterol directly from the blood, after absorption, into the bile.

According to Friedman and Byers[1], since the biliary CHOL represents a quota of the hepatic synthesis of the sterol, its dosage in the bile of rats treated for a certain period with a specific compound can be utilized to estimate eventual pharmacological effects on such synthesis. Without going into the merits of the origins of biliary

cholesterol, which are not yet completely elucidated, we have studied, the effects of several cholesterol-acting drugs employed in a human clinic as cholesterol-lowering substances on the biliary excretion of the sterol in the rat.

METHODS AND MATERIAL

For all the experiments, 350 albino rats of the Morini strain, weighing 250–300 g, were used. In our experiments we used the technique of temporary biliary fistula, as described by Preziosi *et al.*[4–6]

The compounds examined, in the doses marked at the side of each and expressed in mg/kg are: nicotinic acid (NA: 30—85·5—171); 3-pyridineacetic acid (3-PAA : 30—85·5—171); inositol hexanicotinate (IHN : 30—85·5—171); Na-phenylbutyrate (Na-PHB : 25—50—100); betaine phenylbutyrate (betaine—PHB : 25—50—100); 1 : 4-dicaffeylquinic acid or cynarine, true active principle of the artichoke (CYN : 16·6—83—166), and Na-dehydrocholate (Na-DHC : 13·11—65·57—131·14). Reagent-grade compounds were employed. The lowest dose of each compound used by us corresponds, per kilogram, to that advised for human therapy per kilogram, daily. The doses of NA, 3-PAA and IHN are expressed as nicotinic acid, those of Na-PHB and of betaine-PHB as phenylbutyric acid, those of Na-DHC are equimolecular to those of CYN.

All drugs were dissolved in distilled H_2O, with the exception of IHN and CYN, dissolved as described by Donatelli *et al.*[7] and Preziosi *et al.*[6] respectively. The strength of all solutions was calculated in order to inject the required amount of drugs in a total volume of 5 ml/kg.

In a first group of experiments (experiment A) the drugs were administered by intravenous injection. The biliary flow was controlled and measured 1 hr before and 4 hr after the administration of NA, 3-PAA, IHN, Na-PHB, betaine-PHB, CYN and Na-DHC. In the controls, without any treatment, the flow was recorded for 5 hr. In a second group of experiments (experiment B) the method suggested by Byers and Friedman[8] was adopted. This consists in assaying the amount of CHOL emitted in the bile over a period of 24 hr, in rats pre-treated for some days with the drug under test, and comparing the results with those of the controls (untreated animals). The rats were injected by the intraperitoneal route with NA, 3-PAA, IHN, Na-PHB, betaine-PHB, CYN and Na-BHC, for 10 days. On the eleventh day, 14–16 hr after the last injection of the different substances, all rats (untreated controls and treated animals) underwent a temporary biliary fistula, as described above. The bile issuing from the fistula was collected over 24 hr and assayed for CHOL content. During all the experiments, the rats were kept at a temperature of 20–22 °C.

On the samples of bile of each group of animals, collected every hour for 5 hr in experiment A, and for 24 hr in experiment B, the dosage of the total CHOL was carried out by the method of Sperry and Webb[9] with the modifications described by us elsewhere.[4, 5] For the extraction of the CHOL, 1·5–2 ml of bile were used (due to the very small quantity of CHOL present in the bile), which compelled us, in the experiment A, to pool the samples of each group relative to the different times of collection, the quantity of each single sample being insufficient to establish an exact estimation. In order to eliminate biliary chromogens, which we found were capable of remaining adsorbed to the cholesterol digitonide precipitate and thus disturbing the final colour reaction, we proceeded to a first washing of the precipitate with alcohol

acetone, then carried out the subsequent washings, with acetone-ether and ether alone respectively, as described in the original method by Sperry and Webb.[9]

In each case the rate of CHOL was established in milligrams per 100 millilitres (mg/100 ml) of bile. Taking into consideration the volumes of bile obtained, the calculation was carried out of the absolute figures and of the concentrations (mg per cent) of CHOL excreted in the basal hour and in the four successive hours (hours of observation for the controls, post-treatment hours for the rats injected with the drugs), and finally the hourly percentage variations in the quantities of CHOL excreted after the basal hour.

Whenever necessary, the numerical data obtained from the tests were statistically evaluated according to methods suggested by Burn.[10] Values of $P<0{\cdot}05$ were considered to be significant.

RESULTS

1. *Experiment A, single intravenous administration of drugs*

As shown in Figs. 1 and 2 (controls), subsequent to the basal hour a progressive decrease occurs in the biliary flow as well as in the biliary concentrations of CHOL, and of the absolute figures of this excreted with the bile. Regarding the compounds examined, we shall observe the results obtained with NA, 3-PAA and IHN lacking in choleretic effects and those registered with Na-PHB, betaine-PHB, CYN and Na-DHC, all these latter drugs being endowed with intense choleretic action, some very lasting.

NA, 3-PAA and IHN: The comportment of the excretion of bile, expressed in percentage variations in respect to figures of basal hour, proved to be similar in the control rats and in those injected with 3-PAA at all doses tested and with NA and IHN at the highest dose. However, with the lowest doses of the latter two products used, the biliary flow is found to diminish with time more than in the controls. With the exception of the rats treated with NA and the highest dose of those used, there did not appear to be any significant variation in the progressive decreases with time either of the biliary concentration of CHOL or of the excretion of CHOL in absolute figures. In fact the decrease itself appeared, for the lowest doses, slightly accentuated with respect to the controls.

Na-*PHB, betaine-PHB, CYN and* Na-*DHC*. In the rats treated with Na-PHB and in particular, betaine-PHB in the dose of 25–50 mg/kg, a strong choleretic effect is to be observed and there is, as in the controls, a decrease in the concentrations of CHOL in the bile. However, the treated rats differ from the controls by registering an increase in absolute figures of the sterol excreted, due to the larger quantities of bile eliminated for the choleretic effect of the two products. In the rats treated with the two compounds with a dose of 100 mg/kg, however, the controls showed a much more marked decrease in the concentrations of CHOL in the bile emitted following injection of the drugs, and consequently also a greater decrease in the absolute figures of CHOL excreted.

CYN partly opposes (especially at the highest doses and notwithstanding the increased biliary flow determined by such doses) the decrease in concentrations of CHOL in the bile excreted during the 4 hr following the intravenous administration and determines, therefore, at the highest doses, an increased excretion of CHOL in absolute figures.

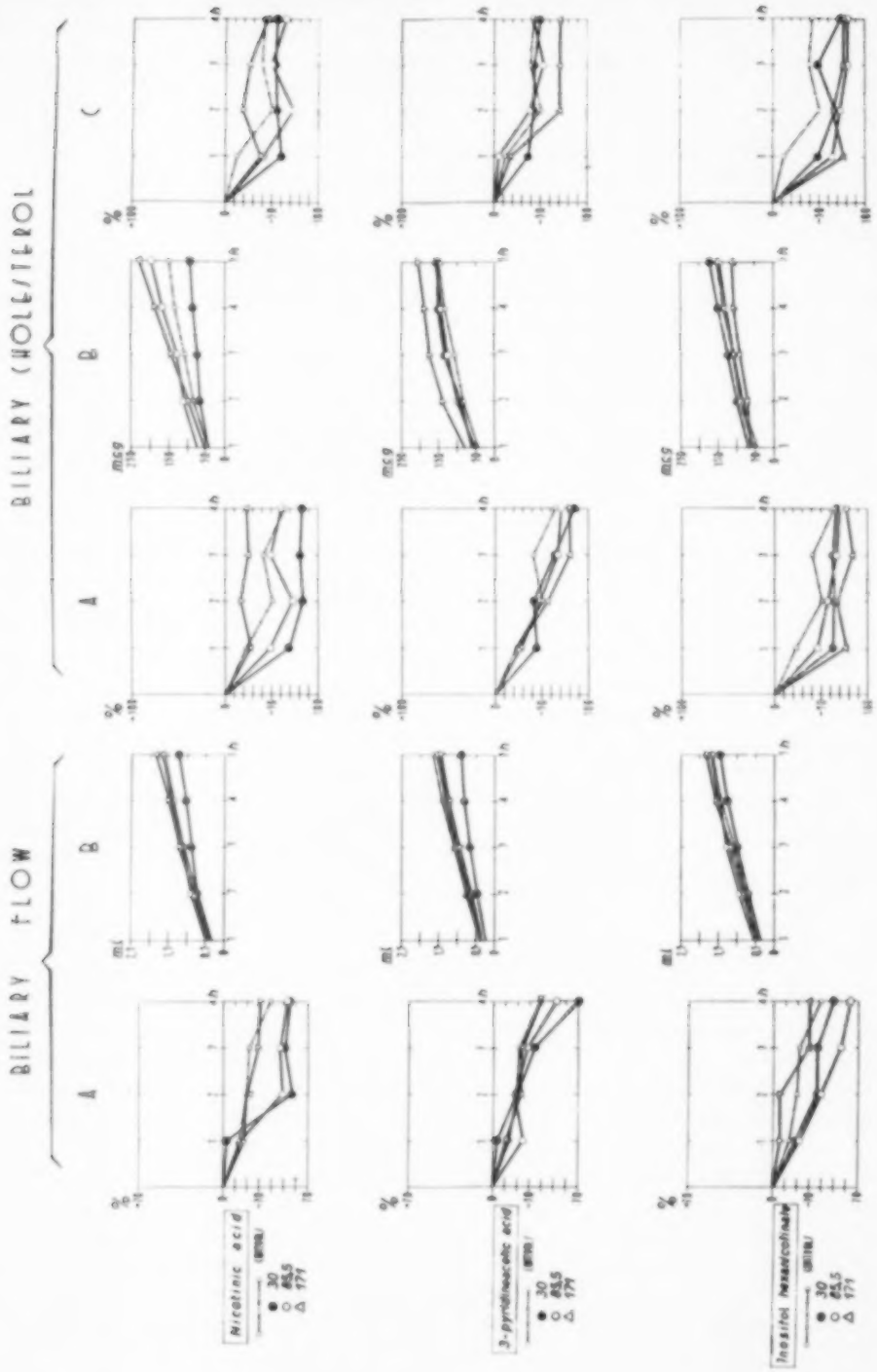
BILIARY FLOW
BILIARY CHOLESTEROL
A
B
C
Nicotinic acid
3-pyridineacetic acid
Inositol hexanicotinate
30
85,5
171

FIG. 1. Effects of nicotinic acid, 3-pyridineacetic acid and inositol hexanicotinate on biliary flow and on biliary excretion of cholesterol.

In the graphs indicated with *biliary flow*, A and B, there are respectively reported, for the three substances examined, the percentage variations of the biliary flow in respect to the figures of the basal hour, that is before treatment, taken as 100 and the biliary flow (expressed in ml) in respect to time obtained adding to the figures of the first hour those of the second hour; to those of the second hour those of the third hour and so on.

In the graphs indicated with *biliary cholesterol* there are respectively reported, for the three substances experimented: in the graph A the percentage variations of the absolute quantities of CHOL excreted by means of the bile in the hours following the administration of the drugs, in respect to the values of the basal hour, taken as 100 (graph A); in graph B the absolute figures of CHOL excreted with the bile in respect to time obtained adding to the figures in the first hour those of the second; to these, those of the third; and so on; in graph C the percentage variations of the biliary concentrations of CHOL in the hours following the basal one, taken as 100, being the figures recorded in this latter.

The doses of the three compounds are expressed as nicotinic acid.

Controls = untreated animals.

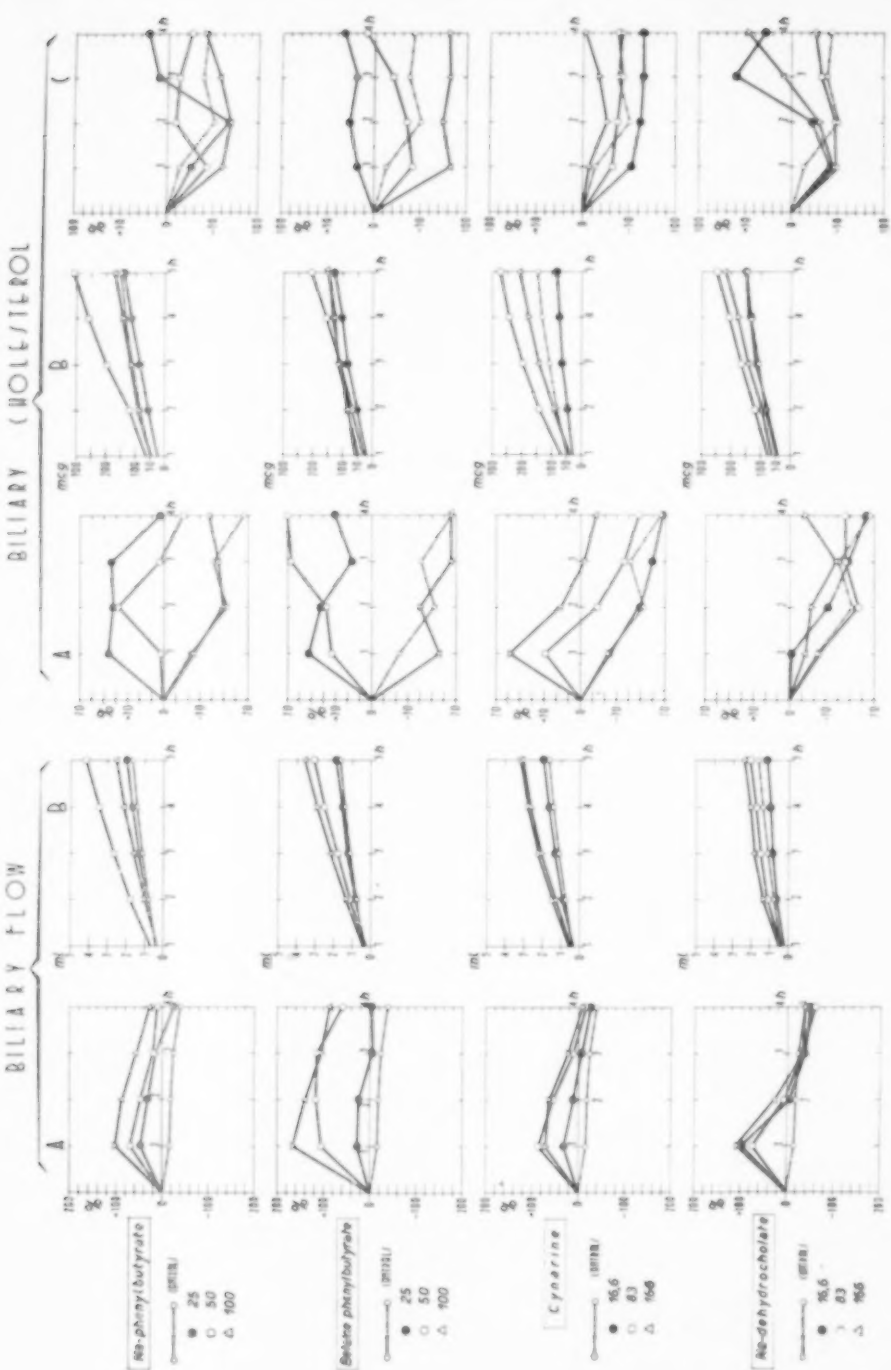
BILIARY FLOW
BILIARY CHOLESTEROL
A
B
C
Na-phenylbutyrate
Sodium phenylbutyrate
Cynarine
Na-dehydrocholate
25
50
100
15.6
83
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FIG. 2. Effects of Na-phenylbutyrate, betaine phenylbutyrate, cynarine and Na-dehydrocholate on biliary flow and on biliary excretion of cholesterol.

In the graphs indicated with *biliary flow*, A and B, there are respectively reported, for the four substances examined, the percentage variations of the biliary flow in respect to the figures of the basal hour, that is before treatment, taken as 100 and the biliary flow (expressed in ml) in respect to the time obtained adding to the figures of the first hour, those of the second; to those of the second, those of the third; and so on.

In the graphs indicated with *biliary cholesterol* there are respectively reported, for the four substances experimented: in graph A the percentage variations of the absolute quantities of CHOL excreted by means of the bile in the hours following the administration of the drugs, in respect to the basal hour, taken as 100 (graph A); in graph B, the absolute figures of CHOL excreted with the bile in respect to the time obtained adding to the figures of the first hour those of the second, to those of the third, and so on; in graph C the percentage variations of the biliary concentrations of CHOL in the hours following the basal one, taken as 100 being the figures of concentration recorded in this.

The doses of Na-phenylbutyrate and of betaine phenylbutyrate are expressed as phenylbutyric acid; the Na-dehydrocholate was administered at doses equimolecular to cynarine ones.

Controls = untreated animals.

In Na-DHC-treated animals, the biliary concentration of CHOL presents a comportment, in the first 2 hr after treatment, not very different from that of the controls. In the first hour, moreover, a positively marked decrease in respect to the figures mentioned in the controls is shown. Since the decrease in biliary concentrations of CHOL takes place during the period of choleretic effect of the product, which lasts nearly 2 hr, there is, in consequence of such a phenomenon and in spite of the reduction of the biliary concentration of the sterol, a reduction (even though slight) of the progressive decrease in the absolute quantity of CHOL excreted by means of the bile. In the third and fourth hours following the administration of the compound, there can be noted, in proportion to a reduction in the biliary flow (even more intense than that noted in the controls at corresponding times), a progressive increase in the biliary concentration of CHOL for which, finally, the excretion of the sterol in absolute figures is comparable, at those times, to those noted in the controls or slightly less.

2. *Experiment B, administration of drugs by the intraperitoneal route for ten consecutive days*

The results of this group of tests are reported in Table 1. Significant variations are not found between differently treated animals (with respect to the control rats) in the quantity of bile emitted for 24 hr and per kg as well as the percentage of CHOL present in the bile and the total quantities excreted of the sterol. Let us mention, however, that only the CYN, at the highest dose of the three examined, reduces the biliary concentration of CHOL, thus confirming what was already noted by us in former research.[4, 5]

DISCUSSION

Among the drugs studied in experiment A, only CYN, at the highest doses examined, and Na-PHB and betaine-PHB, at the lowest doses, increased the biliary excretion of CHOL. The increased excretion of CHOL provoked by CYN may represent a useful factor in clarifying the mechanism of the hypercholesterolemic effects of the product also observed by us in certain experimental hypercholesterolemias[4, 5] and also observed for prolonged treatments at high doses in a human clinic[11, 12] in conditions of deranged cholesterol metabolism and atherosclerosis.

We consider it worthwhile noting that the effect of Na-PHB and betaine-PHB on the biliary excretion of CHOL can differ in the acute experiments, in relation to the dose. In fact, for the lower doses (25–50 mg/kg) practically corresponding per kilogram to those of human therapy per kilogram and daily, there is an increased excretion of CHOL in absolute figures (in the percentage sense a decrease can always be observed, strongly counteracted, however, by the increased biliary flow). At the highest dose, 100 mg/kg, there is a decrease even in absolute figures, there being the strong reduction for such doses of biliary cholesterol concentrations, this time of such entity as not to be compensated by the nevertheless very high increase in the biliary flow. In all probability, for high doses of phenylbutyrates, with the intensification of the phenomenon of inhibition of the hepatic synthesis of the sterol, this phenomenon prevails in respect to the increased biliary excretion, with the result that the bile, even though abundant, contains lesser amounts of CHOL.[13] In spite of the fact that these are also powerful choleretics, this phenomenon does not occur with CYN and Na-DHC even at the highest doses. This variation in action according to the dose of

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TABLE 1. BILIARY FLOW AND BILIARY EXCRETION OF TOTAL CHOLESTEROL IN TEN DAYS TREATED RATS WITH SEVERAL ANTI-CHOLESTEROLEMIC COMPOUNDS

Rats no.	Average weight	Treatment (10 days)		Volume of biliary flow		Total cholesterol content		
		Substance	mg/kg per day i.p.	ml 0–24 hr (± s.e.)	ml/100 g body wt.	mg % (± s.e.)	mg	mg/100 g body wt.
25	230	—	—	6·00 ± 0·935	2·68	15·29 ± 0·709	0·917	0·398
7	254	Nicotinic acid	30	6·93 ± 0·930	2·74	16·91 ± 1·829 $P = 0·2–0·1$	1·221	0·459
7	266	Nicotinic acid	85·5	6·92 ± 0·709	2·60	17·78 ± 0·525 $P = 0·4–0·3$	1·220	0·458
9	280	Nicotinic acid	171	7·52 ± 1·910	2·68	16·46 ± 0·922 $P = 0·3–0·2$	1·237	0·441
7	272	3-Pyridine acetic acid	30*	7·00 ± 0·455	2·57	15·87 ± 0·792 $P = 0·6–0·5$	1·110	0·408
8	250	3-Pyridine acetic acid	85·5	5·66 ± 0·473	2·26	15·93 ± 0·856 $P = 0·6–0·5$	0·901	0·360
6	260	3-Pyridine acetic acid	171	6·15 ± 0·534	2·32	15·23 ± 0·657 $P = 0·7–0·6$	0·936	0·360
5	234	Inositol hexanicotinate	30*	5·50 ± 0·944	2·35	15·89 ± 0·590 $P = 0·9–0·8$	0·873	0·373
5	265	Inositol hexanicotinate	85·5	6·00 ± 0·850	2·64	15·88 ± 0·789 $P = 0·6–0·5$	0·952	0·359
5	248	Inositol hexanicotinate	171	6·00 ± 0·790	2·41	16·15 ± 1·310 $P = 0·6–0·5$	0·969	0·390
10	278	Na phenylbutyrate	25*	7·24 ± 0·663	2·60	18·41 ± 1·600 $P = 0·02–0·01$	1·334	0·479
7	238	Na phenylbutyrate	50	8·00 + 1·021	3·36	18·46 ± 1·191 $P = 0·02–0·01$	1·476	0·620

TABLE 1—*continued.*

Rats no.	Average weight	Treatment (10 days)		Volume of biliary flow		Total cholesterol content		
		Substance	mg/kg per day i.p.	ml 0–24 hr (± s.e.)	ml/100 g body wt.	mg % (± s.e.)	mg	mg/100 g body wt.
8	236	Na phenylbutyrate	100	6·50 ± 0·037	2·75	18·32 ± 1·259 $P = 0·02–0·01$	1·190	0·504
8	272	Betaine phenylbutyrate	25*	8·40 ± 1·042	3·09	18·85 ± 2·382 $P = 0·05–0·02$	1·583	0·582
6	208	Betaine phenylbutyrate	50	7·50 ± 0·707	3·60	16·06 ± 1·410 $P = 0·6–0·5$	1·204	0·578
9	266	Betaine phenylbutyrate	100	8·00 ± 0·670	3·07	16·23 ± 0·870 $P = 0·3–0·2$	1·290	0·484
8	287	Cynarine	16·6	7·00 ± 0·740	2·43	15·01 ± 1·350 $P > 0·9$	1·050	0·356
6	237	Cynarine	83	7·00 ± 0·630	2·99	15·32 ± 1·415 $P > 0·9$	1·072	0·452
6	288	Cynarine	166	6·50 ± 1·340	2·25	13·56 ± 0·660 $P = 0·1–0·05$	0·881	0·305
7	283	Na dehydrocholate	13·11*	7·20 ± 0·420	2·54	16·87 ± 0·629 $P = 0·2–0·1$	1·214	0·428
6	297	Na dehydrocholate	65·7	8·00 ± 0·836	2·69	14·22 ± 0·447 $P = 0·3–0·2$	1·137	0·382
14	288	Na dehydrocholate	131·14	8·60 ± 0·876	2·98	14·46 ± 0·541 $P = 0·4–0·3$	1·243	0·431

i.p. = intraperitoneally.
* The doses of NA, 3-PAA and IHN are expressed as nicotinic acid, those of Na-PHB and of betaine-PHB as phenylbutyric acid, those of Na-DHC are equimolecular to those of CYN.
s.e. = standard error.
P with respect to the controls.

phenyl-ethylbutyrates may explain the different or even opposite effects noted by different authors with phenylethylbutyric acid relative to the biliary excretion of CHOL.[14, 16]

The inability of Na-DHC, among the compounds endowed with a choleretic action examined by us, to determine an increased excretion of CHOL, even if for some of the doses it behaved in the way we observed, confirms the observations of Turba and Piccinini.[17] It is also worthwhile noting that NA is like 3-PAA and IHN, i.e. drugs endowed with marked cholesterol-lowering properties (NA[18]; 3-PAA;[14, 19, 20] IHN[21]), have proved themselves to be incapable of increasing the biliary excretion of CHOL and the biliary flow. The observations made by us confirm the findings of Mainardi[22] and Gunther[23] regarding the inability of NA to modify the biliary flow, and of Friedman and Byers[24] regarding the inability of the acid recorded to favour the excretion of CHOL with the bile; our results, on the other hand, do not agree with those of Parson and Flinn[25], who appear to have found a remarkable capacity of NA of favouring an increased biliary excretion of CHOL.

It must be emphasized again that in the chronic experiments (experiment B), carried out according to the advice of Byers and Friedman,[8] none of the compounds examined, even at much higher daily doses per kilogram than those of human therapy, succeeded in modifying in a statistically significant manner the excretion of CHOL via the biliary route. With the exception of 3-PAA[26] and CYN,[4, 5] the other compounds were never tested by Byers and Friedman.[8] We must therefore conclude, according to Byers and Friedman, that none of the compounds examined (including the phenylthylbutyrates) is able to inhibit the hepatic synthesis of CHOL, with perhaps the exception of CYN ($0{\cdot}1 > P > 0{\cdot}05$) at the highest dose examined, this fact having already been observed by us in former experiments.[4, 5] We postpone, however, such conclusions, since at present a series of investigations is in hand, with the aim of ascertaining the real significance and possibilities of the test advised by the American authors.

Finally we think it worthwhile making note of the possibility (clearly established by the tests carried out by us) that the excretion of CHOL by means of the bile presents variations even in a relatively short time (experiment A) and that such variations, in the sense of an increase in the quota of sterol excreted, can be determined by certain compounds (e.g. phenylethylbutyrates, CYN) active as cholesterol-lowering substances in humans and possessing also marked choleretic action, whilst other powerful choleretics, even though endowed with antiaterosclerotic action[27] even if questionable do not prove to be as active in this sense.

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SHORT COMMUNICATION

Inhibition of the metabolism of hexobarbital *in vitro*

(*Received* 19 *September* 1960)

In a previous report by Fujimoto *et al*[1] it was suggested, as a result of experiments *in vivo* with certain agents which prolong hexobarbital narcosis, that the metabolism of the barbiturate is inhibited by these agents. Experiments involving the perfusion of the isolated rat liver provided further evidence in support of this conclusion. The agents studied were of current pharmacologic interest; these included β-phenylisopropylhydrazine ((PIH) α-methylphenethylhydrazine)*, N-ethyl-3-piperidyl benzilate (EPB) and N-methyl-3-piperidyl-(N′, N′)-diphenylcarbamate (MPDC).† PIH is a strong monoamine oxidase inhibitor.[2] EPB is a hallucinogenic agent described by Abood *et al*.[3]

Since it is known that the liver microsomal system is responsible for oxidation of hexobarbital,[4] it was of interest to determine whether the inhibition of hexobarbital metabolism, as suggested by the *in vivo*-findings mentioned above, could be demonstrated *in vitro*.

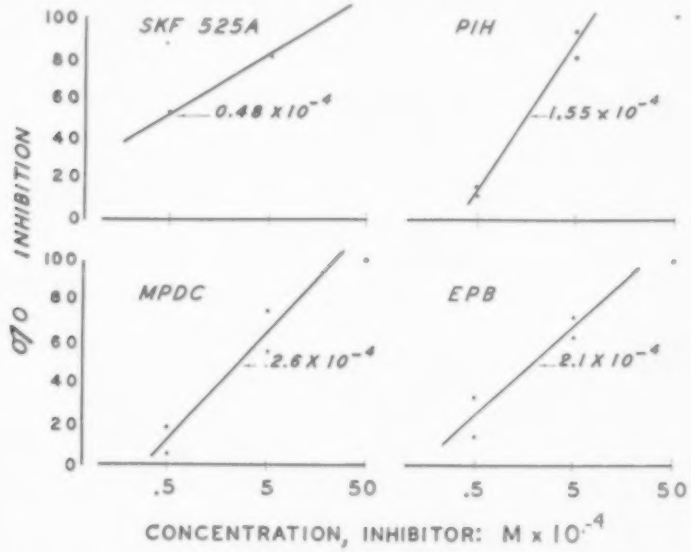

Fig. 1. Inhibition of the metabolism of hexobarbital *in vitro* in a rat liver homogenate supernatant fraction. Concentration of each drug producing 50 per cent inhibition is indicated.

For each drug tested, two adult Sprague–Dawley rats (210–350 g) were stunned and decapitated; the livers were removed immediately, pooled, and homogenized with two volumes of phosphate buffer in a Waring blendor. The supernatant fraction containing the microsomal enzyme system responsible for the enzymic alteration of hexobarbital was then prepared according to the method of Fouts and Brodie[5]. The drug was added directly to the prepared reaction vessels and the degree of

* PIH is also known as JB-516 and is marketed as "Catron".

† EPB is JB-318. MPDC is JB-371. These compounds were obtained through the courtesy of Lakeside Laboratories, Milwaukee, Wisconsin.

inhibition of hexobarbital metabolism was measured by determining the amount of the added hexobarbital remaining in the flasks containing the inhibitor, as compared to that found in the control flasks. Each flask contained 2 μmoles of hexobarbital. Control flasks metabolized hexobarbital at a rate of 1·33 $\pm$ 0·15 (S.D.) μmoles/g/2 hr. The hexobarbital concentrations were determined by the method of Axelrod *et al.*[6] The concentration ranges of drugs were 5×10^{-5} to 5×10^{-3} M. At each level of drug tested, incubations were carried out in duplicate, except for SKF 525–A, with which only single samples were run. This drug had been shown to inhibit this system;[5] thus, it served as a reference drug with which the drugs presently under study could be compared.

The results in Fig. 1 show that the agents PIH, MPDC and EPB, in concentrations slightly larger than that of SKF 525–A, inhibit the liver microsomal system which metabolizes hexobarbital. This inhibition of hexobarbital *in vitro* supports the earlier conclusion that these agents interfere with the inactivation of hexobarbital. Thus the prolongation of sleeping time, higher body concentrations and delay in disappearance from liver perfusate of hexobarbital[1] can be explained by the inhibition of an enzyme system responsible for the metabolism of hexobarbital.

Although the data do indicate the relative potencies of these agents as inhibitors of metabolism of hexobarbital, the present experiments give no information about the specificity of these agents. However, SKF 525–A is known to affect many enzyme systems in the liver, as pointed out by Neubert and Timmler[7]. PIH inhibits monoaminoxidase activity.[2] Our previous work[1] showed that PIH affected not only hexobarbital narcosis but bromsulfalein clearance and thiopental narcosis. EPB affected hexobarbital and thiopenal narcosis but not bromsulfalien clearance. MPDC seemed to be relatively the most specific in inhibiting hexobarbital metabolism in that only hexobarbital narcosis was affected; bromsulfalein clearance and thiopental narcosis were unaffected. Thus it would seem that the least potent compound *in vitro*, MPDC, may on the other hand possess the highest degree of specificity for inhibiting the metabolism of hexobarbital.

Acknowledgement—This work was supported in part by U.S. Public Health Service Research Grant RG–5514.

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BOOK REVIEWS

E. F. ANNISON and DYFED LEWIS: **Metabolism in the Rumen.** Methuen and Co. Ltd., London, 1959. pp. 177, 15s.

A GREAT deal of work on ruminant digestion has been done during the last 20 years and this monograph summarises the biochemical aspects of the subject. The title emphasises the fact that a study of the digestive functions of this organ is really a study of the metabolism of the bacteria and protozoa that live in it. To a large extent the metabolism of these organisms does fulfil the definition of digestion, namely that ingested food is prepared for assimilation by the animal. Since, however, the organisms themselves live on the food eaten by the animal, transformations of food constituents into microbial constituents also occur. The monograph consists of 6 chapters: the first is an introduction which sets out the important anatomical features of the stomach, being marred only by a misleading arrow in the diagram that shows the direction taken by the food through the stomach. This arrow suggests that when food is reswallowed during rumination it passes directly to the 3rd and 4th parts of the stomach, which is not supported by the text. The second chapter deals with the microbiology of the rumen; the third with fermentation of carbohydrates; the fourth with the metabolism of nitrogenous compounds; the fifth with absorption from the rumen; and the sixth with rumen function and dysfunction. Each chapter is annotated and the reference lists, although not exhaustive, give a good introduction to the literature. The text is supported by tables of published experimental results and diagrams.

This monograph is well written, easy to read, and gives a most comprehensive account of the work done in the various aspects of the subject. A clear indication of the ways in which this new knowledge has influenced research on the nutrition of domestic ruminants, on the evaluation of foodstuffs, and on the various disorders that occur in the rumen, or are associated with it, is given. Inevitably there are a few controversial conclusions, but this is only a sign that the subject is rapidly growing, and they are quite overwhelmed by the main body of the book on which substantial agreement has been reached. Consequently it can be recommended confidently as a book which all veterinary and agricultural students should read. It will be useful for those who teach animal nutrition and to those who wish to be well informed on this aspect of biology. The word biology is used because, although the subject matter is largely biochemical, it is treated in a biological manner.

One further matter should be mentioned. This book was written while both authors were on the staff of the Institute of Animal Physiology at Babraham, although the address given to the Preface suggests that this was not so.

A. T. PHILLIPSON

Steric Course of Microbiological Reactions. Edited by G. E. W. WOLSTENHOLME and C. M. O'CONNOR. J. and A. Churchill, Ltd., 1959. Ciba Foundation Study Group No. 2. pp. 115, 12s. 6d.

ONCE the cover of this exciting book is opened there are few faults to be found. The poor printing on the cover is matched by the inaccuracy of its title. Why should a book on microbiological reactions contain a table which compares enzymes from the flounder, sole, lamb, dogfish, grass frog and herring? Although some articles are largely microbiological, the substitution of the word "Biochemical" for "Microbiological" in the title would give a truer picture of the scope of this excellent book.

After NEUBERGER's orientating opening remarks, WESTHEIMER gives an excellent summary of stereospecific reactions. These two articles summarize main themes of the following papers and discussion by treating the biochemical reactions specific for one of the two "a" groups attached to a carbon atom in compounds of the general type Ca_2bc. Citric acid (a = $—CH_2.COOH$), the nicotinamide residue (a = —H) and ethanol (a = —H) are the compounds most thoroughly considered.

WALLENFELS discusses hydrogen transfer by the nicotinamide residue in detail. He uses chemical reactions as models, and some biochemists may wish that the material had been more clearly arranged. Evidence is presented that in those reactions most like enzymic ones, the transfer is of a hydrogen nucleus with an electron pair (i.e. a hydride ion) rather than of a radical or of free electrons. Wallenfels also mentions possible binding mechanisms of the coenzymes with certain dehydrogenases.

KAPLAN follows with a masterly article. After skimming past α-DPN and the neat evidence that it contains the α-ribosidic bond, he reviews L- and D-lactic dehydrogenases from bacteria and animals, and their reactions with coenzyme analogues. Interestingly the two enzymes from *Lactobacillus arabinosus* transfer hydrogen to the same side of the pyridine ring of the coenzymes although to different sides of the pyruvic acid. Finally KAPLAN checks the relationships between fishes, on the basis of the relative behaviour of their heart lactic dehydrogenases with coenzyme analogues.

TALALAY and LEVY lead their readers adroitly through the maze of properties of the steroid dehydrogenases to their determinations of the equilibrium constants of several of the reactions. The surprise is that, whereas in the 5α (rings AB flat) and 5β (rings AB bent) androstane series oxidation of 3α- and 3β-hydroxyls requires, as expected, less energy (by about 1 kcal/mole) when the hydroxyl is axial rather than equatorial, no such difference is found when the molecules bear a $—CO·CH_2OH$ side chain at position 17 at the far end of the molecule. Can this group affect the conformation of ring A three rings away?

PRELOG, in whose honour the meeting was arranged, chooses the reduction of decalones as simpler substrates than steroids. Again we are guided through a variety of microbiological reactions to a simple hypothesis at the end. This states that steric course of the reduction is determined by the larger side of the ketone being compelled to approach from the side away from the $—CO·NH_2$ group of the coenzyme when the oxygen of the ketone points towards the ring nitrogen of the coenzyme. Discussion follows whether this might be a purely steric effect or an orientation of both substrate and coenzyme between polar and nonpolar regions of the enzyme.

The whole booklet gives a fascinating glimpse into an exciting part of biochemistry. The Editors are to be congratulated on the most helpful cross references between the different articles. Could they be asked to read Fowler on hyphens before allowing "nucleic acid-containing particles"? The book is to be praised for the speed of its appearance (the meeting was in March 1959) and for its reasonable price.

H. B. F. DIXON

Medizinische Grundlagenforschung. Edited by K. F. BAUER. Vol. II, pp. 827, 168 DM.

DAS Buch ist der 2 Band einer Schriftenreihe, die den Mediziner in Forschung, Klinik und Praxis über den wissenschaftlichen Fortschritt auf den verschiedensten aktuellen Gebieten in kurzer und verständlicher Form unterrichten soll. Die einzelnen Beiträge sind in sich geschlossen und von hervorragenden Sachkennern geschrieben. Sie nehmen eine Mittelstellung ein zwischen Übersicht und Originalarbeit.

Der vorliegende 2. Band enthält 17 voneinander unabhängige Beiträge. Im ersten Kapitel behandelt O. SCHAUMANN den Schmerz als physiologisches und pathologisches Phaenomen. Der folgende Beitrag von R. GRANIT ist der Steuerung der Körperhaltung durch tonische Hemmungen von der Formatio reticularis gewidmet mit besonderer Berücksichtigung der Gamma-Neuronen. Die "Physiologie der markhaltigen Nervenfaser" ist von H. C. LÜTTGAU, Bern, mit 30 hervorragenden Abbildungen meisterhaft dargestellt. Dieser Beitrag, der die erfreulich eng gewordenen Beziehungen zwischen Physiologie und Pharmakologie zur Grundlage hat, verdient Bewunderung. Schon recht speziell ist das Kapitel von E. C. CROSBY, T. HUMPHREY und M. J. SHOWERS über die supplementären motorischen Rindenregionen. Anschliessend gibt E. HAGEN, Bonn, eine mit vielen guten Abbildungen versehene Übersicht "über morphologische Veränderungen vegetativer Nervenzentren bei pathologischen Vorgäangen". Nach einer kurzen allgemeinen Darstellung der Zellenlehre und der Zellular-Pathologie unter neuen Aspekten von K. F. Bauer und E. MÜLLER berichtet T. ASTRUP über die Fibrinolyse und Thrombolyse. E. RUTISHAUSER, R. LAGIER und E. GRASSET liefern neue Beiträge aus der Arthrose- und Athritis-Forschung. Die Pathologie der Endocarditiden ist von G. BOMPIANI und A. ASCENZI behandelt. Grosses Interesse wird der Beitrag von V. BECKER über Wirkungen des äusseren und inneren Sauerstoffmangels finden. Dasselbe gilt für die Biochemie der Entzündung.

die von R. MEIER (43 Seiten Literatur bei 34 Seiten Text) behandelt wird. Durch Klarheit und Vollständigkeit ausgezeichnet ist die schöne Übersicht über cancerogene Stoffe von N. P. BUU-HOI Anschliessend berichten G. DOMAGK, L. JÜHLING und J. PÜTTER über Probleme der Chemotherapie bei Tumoren besonders mit Bayer E 39 und J. VONKENNEL und M. SCHOOG über die Chemotherapie der Hautkrankheiten. Nach kurzen Beiträgen über Zell-Elektrophorese von G. RUHENSTROTH-BAUER und P. SACHTLEBEN und über Vitamin B_{12}-Analoga von W. FRIEDRICH und K. BERNHAUER schliesst das Buch mit einer recht interessanten Abhandlung von R. HAASE: "Strukturbildung in der Natur und der 2. Hauptsatz der Thermodynamik" und betont damit wohl bewusst die naturwissenschaftliche Grundeinstellung.

Die wissenschaftliche Qualität der einzelnen Beiträge ist durchweg gut. So gibt das Buch dem Leser die willkommene Möglichkeit, sich in kurzer und klarer Form von berufenen Sachkennern über die wichtigsten Fortschritte der Forschung unterrichten zu lassen. Ein solches Buch hat gefehlt, so dass man den Autoren, dem Herausgeber und dem Verlag für ihre Arbeit nur danken kann.

H. DRUCKREY

Ciba Foundation Symposium on Regulation of Cell Metabolism. Edited by DR. G. E. W. WOLSTENHOLME. J. and A. Churchill Ltd., London, 1960. pp. 387, 52s. 6d.

THIS is an account of an enthralling Symposium planned and directed by Sir Hans Krebs with the cooperation of Professor Dickens as a result of a conversation between Professor Martius, Professor Lynen and Sir Hans Krebs. It is the result of papers by sixteen authors followed by an account of the discussion of these papers. Thirty-one persons attended and took part in this Symposium.

It commences with a Chairman's introductory address on the rate-limiting factors in cell respiration in which there is a consideration of how tissues such as cardiac muscle, showing great variations in the rate of respiration, all the changes from rest to activity, nevertheless can manage the consequential variations in a way in which factors other than hormonal may be involved. It deals with the reactions between oxygen and substrates involving such factors as iron porphyrins, flavoproteins and pyridine nucleotides. It points out the importance of feed-back systems in control. It is interesting that this mechanism has now become generally accepted in biochemistry, as it is one that was clearly enunciated by Sherrington in regard to reflex activity many years ago. The address by Krebs is followed by one by Siekevitz on the meaning of intracellular structure for metabolic regulation. He brings out well the various problems involved in the recent knowledge of the structure of mitochondria and of cytoplasm, in pointing out the modern views on the division of cytoplasm into numerous compartments. Clearly in matters of regulation the incidence of these barriers must be very thoroughly considered, and a similar aspect is discussed by De Duve.

E. C. Slater and W. C. Hülsmann deal with the respiratory chain and the relation to this of ADP. Aspects of control of oxygen utilisation are discussed by Britton Chance with a full account of his fascinating techniques for investigating reactions occurring in seconds, and his solution of many simultaneous equations.

A. Lehninger and colleagues discuss control points in phosphorylating respiration and the action of a mitochondrial respiration release factor. In this they delineated the effects of various uncoupling reagents. F. Dickens and colleagues have made a large contribution to the discussion, on various oxidative pathways of carbohydrate metabolism dealing, in addition to normal pathways, with the glucuronate pathway and the pentose phosphate pathway. Lipmann and colleague discuss glycogen synthesis in muscle. Martius deals with electron transport and is followed by a series of papers upon the control of glycolysis by Racker, Potter, Lynen and Holzer. It would be invidious to pick out special points of interest in this wide appreciation of the subject; that on triphosphopyridine nucleotide by Potter and Niemeyer, and the regulation of the Pasteur effect by phosphorylation reactions in relation to the exhaustion of the inorganic phosphate by Lynen seem very significant.

The Symposium finished with papers on the automatic adjustment mechanisms in bacterial cells by Dean and Hinshelwood, and control of growth of the bacterial cell by Magasanik *et al.*

The theme of the Symposium in its insistence upon looking at the reactions in the cell as a whole is admirable and provides much food for thought. In the past biochemists have been concentrating naturally upon the behaviour of isolated enzymes. The time has now come when a more forward attempt may be made to put these together and an oversight of these papers makes fairly clear what a

long way we shall have to go before we understand them in all their heterogeneity. The discussions which are also printed are informative and more valuable than the reviewer has often felt in the past. It is possible that these might still be edited more thoroughly with an improvement in the presentation.

Other biochemists could possibly have been included, but some limitation was necessary.

The book which is well produced represents a milestone in biochemical thought, and justifies the hope of Sir Hans that it will be regarded as a pioneer book. The reviewer was only surprised by one challenging sentence to the effect that hormones usually (though not always) act through direct effect on an enzyme. This would not be considered as proved by a majority of biochemists; but they should certainly make a point of reading this symposium.

RUDOLPH A. PETERS

Symposium on Protein Structure Edited by A. NEUBERGER. Methuen & Co., Ltd., London, 1958. Price 45*s*.

THE meeting which gave rise to this volume, held in Paris, 1957, had as its original intention a discussion of the question of purity as it applies to proteins. In the event it covered most aspects of protein chemistry. The gradual enlargement of the scope of the symposium may explain the wide differences in character between the different contributions which range from extensive review articles through more usual symposium papers to detailed accounts of particular experiments. But it has also meant the inclusion of so many topics that all interested in this field will find something of value and interest.

It contains several methodological papers under the heading—general problems and methods—which include a stimulating paper on deuterium exchange by the late Professor Linderstrøm-Lang, and an excellent review by Tanford on protein configurations in solution. The section on specific proteins contains many contributions of great interest, in particular, the accounts of Hirs, Stein and Moore, and by Anfinsen, on the elucidation of such a large fraction of the structure of ribonuclease. Papers on recent developments concerning myoglobin, haemoglobin and tobacco mosaic virus include reviews of the advances in X-ray analysis of these proteins. The paper by Kendrew gives a clear picture of the methods which have since given his model of myoglobin. The rapid progress in the chemistry of the proteolytic enzymes is well covered.

Some miscellaneous observations on a diverse range of subjects comprise the last section. These include the sequence studies on lysozyme by the late Professor Fromageot and one extensive review by Professor Li of the species variation and structures of some of the pituitary hormones.

To one on the fringe of this subject this volume conveys clearly the great interest and pace of work in this field: to the specialist despite the great uneveness in the methods of presentation there is much of interest in this book.

K. A. STACEY

The Thyroid Gland (N. B. MYANT Editor) British Medical Bulletin Vol. 16, No. 2, The British Council, London, May 1960, 20*s*.

THE articles are of the high standard set by previous issues of the Bulletin, and the choice of contributors is a guarantee of their authority. There is, naturally, a clinical orientation, the opening article being on Deafness and Thyroid Dysfunction (W. R. TROTTER); among the topics dealt with are diagnostic use of radio-iodine, auto-immunity, and interrelationships between the thyroid and the central nervous system. Scattered among such articles, in somewhat random order, are articles on more basic topics such as hormone biosynthesis (R. PITT-RIVERS). The latter article lacks formulae and a summary. Bioassay methods are nowhere considered, except in the excellent article by H. D. PURVES and D. D. ADAMS on thyroid-stimulating hormone; this article is marred only by a confusing opening sentence in the section on exophthalmos.

The absence of any articles on actions of thyroid hormone, at the tissue level, is a serious omission, particularly since there is an extensive and confusing literature on this question. It would, moreover, have been advantageous to give at the outset a "refresher" article, surveying established facts on the nature and actions of thyroid hormones (e.g. the role of thyroglobulin) and attempting to put the succeeding articles in perspective. The Bulletin is, then, not ideally suited to the hypothetical "general reader" mentioned in the Introduction.

E. REID

E. H. RELYVELD: **Toxine et antitoxine diphtériques. Etude immunologique.** Actualités scientifiques et industrielles: 1278, Hermann, Paris, 1959. 164 pp, 58 fig., 33 tables.

THIS monograph describes original experiments conducted to study the much discussed reactions between diphtheria toxin and antitoxin. In addition, each chapter contains a historical introduction and several useful techniques are fully described and discussed.

Crude diphtheria toxin (culture-filtrate), and therefore the anatoxin prepared from it, contains a large number of antigens besides the specific toxin. Corresponding anti-sera contains many "accessory antibodies" in addition to "antitoxin", this name being used to designate antibodies specifically reacting with the toxin, either to promote its precipitation or its neutralization.

A new semi-synthetic medium, containing casein hydrolysate, and optimal conditions for toxin production are described as well as two methods of toxin purification, one making use of the classical salting-out method, the other resorting to chromatography on calcium phosphate. A crystalline toxin titrating from 3·000 to 3·100 FU/mg N was obtained as well as a correspondingly pure anatoxin.

Tested by several methods of gel-precipitation, the purified toxin gave only one line of precipitation in response to homologous anti-serum as well as to anti-sera prepared with anatoxins of lesser purity and containing a number of accessory antibodies. Likewise, anti-serum against purified anatoxin gave a single line of precipitation when allowed to react in agar with crude toxin.

At the beginning of immunization in horses, diphtheria antibodies are associated with the γ-globulins and such anti-sera give a bell-shaped quantitative precipitation curve (type "rabbit). On the contrary, at the end of the hyperimmunization, diphtheria antibodies are associated with the β-2-globulins and the quantitative precipitation curve shows a rectilinear part and solubility of the precipitate in excess antibody (type "horse"). Under proper conditions, and with the help of ^{131}I, it was shown that antibodies, synthesized in response to the injection of pure anatoxin, can be of several kinds, some being able to flocculate without hardly neutralizing, while others neutralize without precipitating and others still act both ways. However, a study of the reactions of anti-serum with pure toxin partly degraded by a protease indicates that the toxin molecule is a single entity, provided with several determinants groups, each of which being able to induce the production of a distinct antibody, but only one of which being related to toxicity and therefore able to induce the production of neutralizing antibody.

M. WELSCH

L. REY: **Conservation de la vie par le froid.** Actualités scientifiques et industrielles: 1279, Hermann, Paris, 1959. 167 pp, 84 fig.

SEVERAL chapters of this monograph deal with the physico-chemical processes intervening during the congealing of biological material to low temperature and with the description of techniques and apparatus used to achieve that aim.

From the biological point of view, original experiments are reported showing that, under proper conditions, heart tissue from the chick as well as skin from the rat or mouse, can be stored at low temperature (dry ice: -79 °C; or liquid nitrogen: -196 °C) for an indefinite length of time without showing, upon correctly performed re-heating up to body-temperature, any loss of their physiological activities.

In order to obtain such results, the tissues must be impregnated with glycerol before being cooled. There exists an optimal concentration of glycerol, as well as an optimal length of time for impregnation, which have to be found for each particular material under study.

The range of temperature between 0 ° and -50 °C is the most noxious to the tissues and therefore re-heating must be performed as quickly as possible.

The protection afforded by glycerol is explained by the following observations. Between 0 ° and -20 °C, it slows down the rate of propagation of the crystalline front, diminishes the size of the crystals, favours surfusion and has a tendency to maintain osmotic equilibrium while water is converted to ice. Between -20 ° and -80 °C, its presence gives more regularity to the process of crystallisation and favours the formation of vitreous structures.

The temperature of storage must be below −60 °C for tissues impregnated with Eagle's solution containing less than 45% glycerol and below −130 °C whenever the concentration of glycerol is higher.

Practical applications (tissues and organ banks) and the possibility of congealing and storing whole animals without harm are discussed.

M. WELSCH

Antibiotics in Medicine. L. P. GARROD (Editor). British Medical Bulletin **16**, No. 1, 1960. The British Council, London, 1960. 20s.

THE monograph: *Antibiotics in Medicine*, prepared under the eminently competent scientific editorship of Professor L. P. Garrod, devotes an introduction and fifteen papers, written by well-known specialists to the basic and practical problems of antibiotics. It will be of the highest value to the clinician wishing to make a rational therapeutic and prophylactic use of the numerous now available anti-infectious drugs.

Four chapters are of especial interest to the biochemical pharmacologist.

In the first one, "Chemistry and classification of antibiotics", E. P. Abraham and G. G. F. Newton, show how it is now possible to group the many known antibiotics into natural families on the basis of either their chemical structure or the mechanism of their biosynthesis.

The second one, by E. F. Gale, deals with the "Selective toxicity of antibiotics". It gives a clear and up-to-date picture of what is known of the modes of action of antibiotics at the molecular and cellular levels: inhibition of cell-wall synthesis by penicillin, cycloserine and bacitracin, gross cell-permeability alterations by surface-active antibiotics such as tyrocidin and polymyxin, interference with protein synthesis by chloramphenicol and the tetracyclines.

In the third one, "Drug resistance and mechanisms for its development", M. R. Pollock, first briefly describes the biochemical peculiarities that can be held responsible for microbial drug-resistance. He next discusses the respective roles of biological mechanisms that can conceivably induce more or less stable changes in individual bacteria and therefore, thanks to natural selection, promote population changes: enzymic induction and genic alterations such as gene mutation and gene recombinations of various kinds.

The last one, by J. M. Robson and G. A. H. Buttle deals with "The pharmacology of antibiotics". Perforce, since our knowledge is yet rather limited, the biochemical aspects are less stressed than in the other papers.

M. WELSCH

Glutathione. E. M. CROOK (Editor). Biochemical Society Symposium No. 17. Cambridge University Press, 1959. 116 pp., 15s.

EVER since Hopkins isolated pure glutathione nearly 40 years ago, the elucidation of the function of this ubiquitous substance has posed considerable problems. Some of the latest views on possible roles for glutathione were discussed at a Biochemical Society Symposium held in London in February, 1958.

The first two articles deal with the chemistry and methods of determination and are by F. A. Isherwood on the "Chemistry and biochemistry of glutathione" and by C. G. Thomson and H. Martin on "Techniques for determining glutathione in animal tissues". The next two sections are concerned with possible metabolic roles for glutathione in plants and animals: "Enzyme systems associated with the oxidation and reduction of glutathione in plant tissues" by L. W. Mapson and "Glutathione metabolism in animals" by P. C. Jocelyn. In these articles particular reference is made to the oxidation/reduction of glutathione, its coupling with TPN and with vitamin C, and to the role of glutathione as a coenzyme.

The remaining three sections are concerned with more specialized aspects of glutathione metabolism: "Glutathione and neural tissues" by H. McIlwain, "Glutathione and its analogues in the lens" by S. G. Waley, and "Thiols and radiation damage" by D. B. Hope. The latter article, as its

title suggests, includes a general discussion on —SH compounds, including —SH proteins, in their controversial role as primary targets in radiation damage.

The problem of collating the various possible functions of glutathione is clearly not yet finally solved but considerable progress has been made. The symposium may be strongly recommended to the many biochemists who must be interested in the vital functions of —SH compounds.

E. D. WILLS

G. F. GAUSE. **The Search for new Antibiotics** Yale University Press, 1959, pp. 97, $4.75.

THE problem of coping with an expanding scientific literature is further complicated by the need for making up a deficit in our knowledge of Soviet research of the past two decades. Books such as GAUSE's will acquaint American and European scientists with the achievement and orientation of Russian Institutes. A lecture delivered at Yale University in 1959 forms the basis of this volume. It is a summary statement of some work at the Institute of Antibiotics in Moscow relating to the ecology of antibiotic-producing microbial species, and a more detailed account of the use of mutants with impaired respiration in the selection of anti-neoplastic chemical agents. GAUSE presents evidence that mutant microorganisms with deficient respiration and altered cytochromes are uniquely sensitive to carcinostatic chemicals. This relationship permits a screening technique for antibiotic and synthetic chemicals which is correlated with the effectiveness of the compounds on mammalian tumors. Underlying this work is the postulate that mutant microorganisms with impaired respiration are the biological equivalents of mammalian neoplastic cells. The utility of the empirical relationship remains to be determined, and the biochemical mechanisms which define the relationship are described only in general terms within the restricted parameter of respiration. However, this provocative essay gives the experimental basis of a development in screening technology which may assume considerable importance in the search for agents which selectively inhibit neoplasms.

C. E. CARTER

E. KELEMEN: **Permeability in acute experimental inflammatory oedema in the light of the action of salicylates**. Publishing House of the Hungarian Academy of Sciences, Budapest, 1960. 256 pp.

KELEMEN étudie depuis 10 ans les facteurs qui conditionnent le développement de l'oedème provoqué par l'administration dans la patte du rat d'un extrait de testicule, riche en hyaluronidase. La pathogénie exacte de cet oedème reste encore ignorée. Elle ne dépend pas d'une libération ni d'histamine ni de 5-hydroxytryptamine, mais bien d'une atteinte primitive des capillaires et des membranes basales.

L'action des salicylés sur le développement de l'oedème est conditionnée par la présence des glandes surrénales. Ainsi les salicylés agissent par l'intermédiaire d'un facteur hormonal, encore mal défini. Des interrelations entre cortex et médulla sont en outre démontrées.

L'extension de ces conclusions à la thérapeutique clinique par salicytés à fortes doses semble malaisée. Bien que l'intoxication aiguë par salicylates entraîne certainement une réponse corticosurrénale, pour Kelemen pareille stimulation ne paraît pas survenir lors des cures chroniques.

Outre ces facteurs endocriniens, des modifications de la circulation locale, du métabolisme tissulaire *in situ*, et de l'équilibre hydro-minéral, l'administration d'adrénaline (et non de noradrénaline) peuvent également influencer le développement de l'oedème.

L'administration des salicylés influence chacun de ces facteurs. Ces altérations d'origine pharmacodynamique ont été considérées en détail, en particulier chez l'homme. De l'examen de ces données, l'auteur établit qu'il existe une relation entre les propriétés anti-inflammatoires (en particulier des salicylés) d'une part et d'autre part ce qu'il définit comme l' "hypergie", c'est-à-dire une altération des échanges hydro-minéraux associée à une utilisation tissulaire insuffisante d'énergie métabolique.

Le travail de Kelemen représente une contribution importante à la connaissance de la pharmacologie des salicylés et des mécanismes de l'inflammation.

L'action des salicylés apparaît fort complexe, s'expliquant par une stimulation cortico-surrénalienne, par des interactions médullocorticales et par des facteurs métaboliques tissulaires plus généraux, tels que l'utilisation inadéquate des composés phosphorylés à haute teneur énergétique.

La mise en évidence d'une perturbation des processus métaboliques comme condition essentielle de l'apparition des réactions inflammatoires découle de cette analyse. Les troubles de la perméabilité s'expliqueraient par une atteinte cellulaire primordiale, dont le déterminisme humoral ou endocrinien semble très secondaire.

Les conceptions de Kelemen s'avèrent originales et, par là même, prêteront sûrement à discussion. Rappelons simplement que pour Roskam et son école, la salicylothérapie en clinique humaine dépend avant tout d'une stimulation cortico-surrénale, qui a échappé aux moyens d'investigation mis en oeuvre jusqu'ici par l'auteur hongrois.

Mais quelles que soient les divergences entre les opinions de l'auteur et celles des autres chercheurs, les travaux de Kelemen ont le mérite d'envisager les phénomènes inflammatoires sous un aspect biochimique et physiopathologique nouveau qu'il ne faudra plus, désormais, méconnaître. Il faut cependant regretter que l'auteur ait adopté une présentation trop analytique de ses recherches, laissant ainsi au lecteur le soin d'un effort de synthèse parfois malaisé.

J. LECOMTE

E. GURR: **Encyclopaedia of Microscopic Stains.** Williams & Wilkins, Baltimore, Maryland, 1960. 498 pp. $18.50.

RIDING the crest of the present popular wave of books devoted to methodology comes this one which is an "encyclopaedia" (quotes mine) of stains and dye stuffs used in histological practice. The book is adequate in relation to its intended purposes—a reference and laboratory guide on the application, structure, composition, molecular weights and properties of a large number of dyes and other reagents used in microscopic anatomy. However, the position of the reviewer is that of one who feels inundated by the wave and hence the following reaction:

As a laboratory guide there are already too many books currently available that surpass it. About one-half of the space of the book is devoted to formulae of dyes and reagents. These can be found elsewhere. And as concerns the interest and importance of structural formulae to the morphologically schooled audience for which it is intended, unfortunate as it may be, this score is almost zero. Is the book a catalog for the Michrome Laboratories? All of the dyes and reagents except a very few like iodine are given a Michrome number and only in a few cases are adequate synonyms given for the names of dyes. Dyes are listed alphabetically instead of being classified by chemical structure. Diazonium salts are listed primarily by Michrome number; synonyms and formula are given secondarily. Like the dyes that have no Michrome number, other diazotates are not worthy of listing. Tetrazolium salts are inadequately handled; only three are mentioned and these do not include Nitro B.T., the most useful and popular one. Shall I go on—or you pays your money and you takes your choice.

RUSSELL J. BARRNETT

M. STACEY and S. A. BARKER: **Polysaccharides of Micro-organisms.** Clarendon Press, Oxford, 1960, ix 228 pp.

THE increasing importance attributed to polysaccharides in various fields of microbiology fully justifies the publication of a book gathering and co-ordinating the available information on those substances which, at the present time, is widely scattered in a large number of scientific journals. Stacey and Barker's book, a concise compendium of our knowledge of microbial polysaccharides, written by fully competent chemists whose personal researches have substantially added to that knowledge, will therefore be welcomed by all microbiologists, although it cannot be expected to include the latest developments of a rapidly growing field of investigation.

The first five chapters deal adequately, albeit summarily, with general properties: nomenclature of carbohydrates, components of polysaccharides and biosynthesis, functions of polysaccharides, methods for isolation and, finally, for structural determination of polysaccharides.

The last seven chapters are devoted to a detailed study of the polysaccharides produced by various groups of micro-organisms: Rickettsiae and viruses, Gram-negative bacteria, Gram-positive bacteria, higher bacteria, moulds, yeasts and yeast-like fungi, Protozoa. They give, in a condensed form, a large amount of valuable chemical and physical data with full reference to original papers and will, therefore, be a very useful source of information for the specialist. So will be Appendix I which lists the physical constants of the monosaccharide constituents of microbial polysaccharides. A well prepared subject index makes the book easy to consult.

The reviewer feels less happy about some statements that are, however, of a microbiological rather than of a biochemical nature. For example, he cannot agree that: "generally speaking Gram-negatives produce highly potent immunizing fractions and are resistant to many antibiotics while Gram-positives produce relatively transient immunizing fractions and are highly susceptible to antibiotics" (p. 23), or that: "In some Gram-positive cocci (e.g. *Pneumococci*), deoxyribonucleic acid is a *surface* (reviewer's italics) component playing a highly important role in specific polysaccharide synthesis" (p. 23). He also believes that it cannot be stated as a *general* rule that: "The presence of a capsule is one of the conditions for the virulence of a cell" (p. 24).

M. WELSCH

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Papers to be published in future publications

E. Paroli and P. Melchiorri: Urinary excretions of hydroxysteroids—I. 17-ketosteroids and aldosterone in rats during a cycle of treatment with morphine.

E. Paroli and P. Melchiorri: Inhibitory effects of morphine on metabolism of adrenal and testicular steroids.

P. Alexander and Z. B. Mikulski: Differences in the response of leukaemia cells in tissue culture to nitrogen mustard and to dimethyl myleran.

J. D. Gabourel: Anticonvulsant properties of diacetylmonoxime (DAM).

Tibor Zsolnai: Versuche zur Entdeckung neuer Fungistatika—II. Nitro-verbindungen.

E. Bueding, E. Kmeteck, C. Swartzwelder, S. Abadie and H. J. Saz: Biochemical effects of dithiaznine on the canine whipworm, *Trichuris vulpis*.

J. Lecomte and E. Schoffeneils: Action du 2-PAM et des inhibiteurs de l'acetylcholinesterase sur le choc anaphylactique du lapin.

D. P. Wallach: Studies on the GABA pathway—I. The inhibition of gamma aminobutyric acid—alpha ketoglutaric acid transaminase *in vitro* and *in vivo* by U–7524.

J. E. Casida: Specificity of substituted-phenyl phosphorus compounds for esterase inhibition in mice.

Thomas H. Maren, Bettie Robinson, Roger F. Palmer and Mary E. Griffith: The binding of aromatic sulfonamides to erythrocytes.

Robert C. Wood, Robert Ferone and George H. Hitchings: The relationship of cellular permeability to the degree of inhibition by amethopterin and pyrimethamine in several species of bacteria.

Kenneth E. Moore and Theodore M. Brody: The effect of triethyltin on oxidative phosphorylation and mitochondrial adenosine triphosphatase activation.

Kenneth E. Moore and Theodore M. Brody: The effect of triethyltin on mitochondrial swelling.

Karl A. Schellenberg and G. Robert Coatney: The influence of antimalarial drugs on nucleic acid synthesis in *Plasmodium gallinaceum* and *Plasmodium berghei*.

E. D. Wills: Effect of unsaturated fatty acids and their peroxides on enzymes.

Henry G. Mautner and Julian J. Jaffe: 6-Selenoguanine (2-amino-6-selenopurine). Synthesis and biological studies.

Paul Bijvoej and Harris Busch: Effect of 5-bis (2-chlorethyl) aminouracil on incorporation of uracil-2-^{14}C into RNA of tumour-bearing rats.

A. Chari-Bitron: A study of the toxicity and the biochemical effects of monofluoromalonate.

Henrik Wallgren: Effects of acetylcholine analogues and ethanol on the resaration of brain cortex tissue *in vitro*.

W. N. Aldridge and J. M. Barnes: Neurotoxic and biochemical properties of some tri-aryl phosphates.

J. J. Roberts and G. P. Warwick: The mode of action of alkylating agents—II. Studies of the metabolism of myleran. The reaction of myleran with some naturally occurring thiols *in vitro*.

Biochemical Pharmacology, 1961 Vol. 5, pp. 275–282. Pergamon Press Ltd., Printed in Great Britain.

DIFFERENCES IN THE RESPONSE OF LEUKAEMIA CELLS IN TISSUE CULTURE TO NITROGEN MUSTARD AND TO DIMETHYL MYLERAN

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(*Received* 25 *June* 1960, *in revised form* 20 *July* 1960)

Abstract—Two chemically different types of biologically active (radiomimetic) alkylating agents, i.e. nitrogen mustard (HN2) and dimethyl myleran, are shown to produce very different patterns of response in leukaemia cells grown as isolated cells in tissue culture. HN2 immediately stops division, while following dimethyl myleran the cells divide at least once before growth stops. The appearance of the cells is also different as is the shape of the curve relating dose with survival (i.e. ability to proliferate indefinitely). For HN2 the relationship between log survivors and dose is nearly linear, whereas with dimethyl myleran there is a marked threshold. It is suggested that the initial chemical reactions that are responsible for these effects on growth are different possibly because different sites are alkylated. The effects of dimethyl myleran simulate more closely those of X-rays than do those of HN2.

THE radiomimetic activity (i.e. the ability to act as a mutagen and carcinogen, to inhibit cell division, to kill lymphocytes and to cause chromosomal aberrations by acting on the cell during interphase) of the mustards, ethyleneimines, epoxides and esters of methane sulphonic acid is undoubtedly associated with their ability to alkylate, under physiological conditions, molecules within the cell.[1] There are however, many sites available for alkylation and it is possible that different alkylating agents may show a different pattern of reaction and the biological effects they produce will not be identical. The striking similarity in the biological end-effects made it tempting to postulate an identical mechanism at the cellular level for all these substances and to ascribe differences in their effects in mammalian systems to processes of transport and distribution.[2, 3] On the other hand, Elson[4] has consistently advocated that the differences in the blood picture seen after exposure to myleran and nitrogen mustard indicated a difference in mechanism at the cellular level and this view found support in a quantitative study of the bone-marrow[5] of treated rats. Alexander and Lett[6] were led to the same conclusion from biochemical investigations on the effect of these two types of alkylating agents.

In the present investigation the effect of HN2, ($CH_3N.(CH_2CH_2Cl)_2$), and dimethyl myleran, ($CH_3SO_2.O.CH(CH_3).CH_2.CH_2.CH(CH_3).O.SO_2.CH_3$), was studied on the multiplication of mouse leukaemia cells growing as isolated cells in tissue culture.[7] Dimethyl myleran was chosen instead of myleran, ($CH_3.SO_2.O.(CH_2)_4.O.SO_2CH_3$), because its rate of reaction is much greater and its half life under physiological conditions is 37 min[8] as opposed to many hours for myleran.* In its

* A direct comparison is not possible since dimethyl myleran reacts by an SN1 mechanism, so that its rate of disappearance by alkylation or hydrolysis is independent of the presence of dissolved substances, while myleran reacts by an SN2 mechanism when the rate depends on the amount of alkylatable substrate present.[1]

biological effects dimethyl myleran is extremely similar to myleran[9] and shows the same selectivity for myeloid cells that distinguishes the latter from mustards. The half-life of HN2 in the tissue culture medium is less than $\frac{1}{2}$ hr. The rapid action of these substances ensures that the time of treatment of the cells (i.e. the time they are exposed to alkylation) is relatively short and eliminates the need to wash the cells free from reagent.

EXPERIMENTAL

L5178 Y Lymphoblasts, strain specific to DBA/2 mice (originating as a cloned derivative of an ascitic form of L 5178 supplied by L. W. Law of the National Cancer

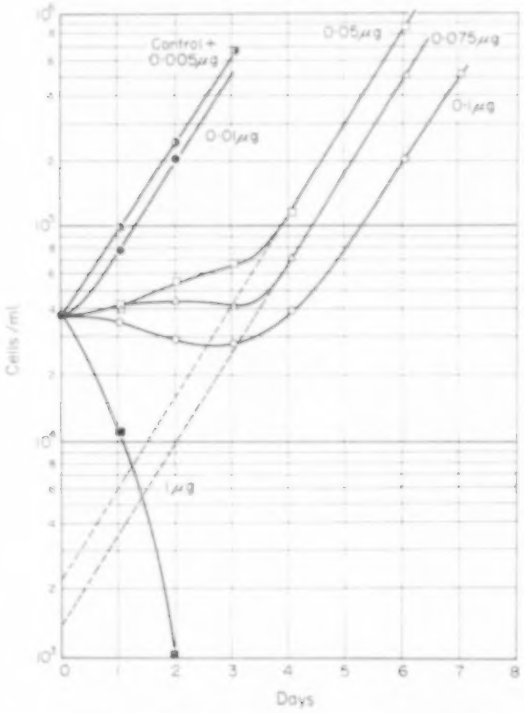

FIG. 1. Growth of leukaemia cells in culture containing different quantities of HN2 $(CH_3.N.(CH_2CH_2Cl)_2$. The concentrations are in μg HN2/ml. The dotted line shows the method of obtaining the fraction of cells of the original inoculum that retain capacity for indefinite proliferation.

Institute, Bethesda) were grown in the medium and by the techniques developed by Fischer.[7*] The cells were periodically transplanted into mice to ensure that they retained their strain specificity. In culture, they grow almost wholly as near diploid cells with a generation-time that varied from 12 to 18 hr, depending on the batch of medium.

In most of our experiments an initial inoculum of from 2 to 10 $\times$ 10^4 cells/ml were used. The culture was diluted when the population approached 10^6 ml. Figs. 1 and 2

* We are greatly indebted to Dr. G. A. Fischer of the Department of Pharmacology, Yale University School of Medicine, for giving us a culture and for much help.

show that the rate of growth of the untreated cultures is accurately logarthmic. Cells were counted in a haemocytometer. The distribution of cell sizes was determined with a "Coulter Counter" in which changes in electrical resistance are used to count and to size particles in suspension.

RESULTS

Growth curves

Comparison of Figs. 1 and 2 shows a most striking difference between the action of these two cytotoxic agents. HN2 stops cell division immediately. Microscopic examination of the culture in the first 24 hr fails to reveal any cell degeneration and the constancy of the cell numbers can therefore be attributed to cessation of division and is not due to compensation of reproduction and distribution. After 40 hr cell degeneration is seen and the cell count now represents a balance between degeneration and production of new cells. With dimethyl myleran, even with very substantial doses, at least one division occurs before growth ceases in the cultures. With low doses of dimethyl myleran, the rate of cell division is initially slowed down to a small extent

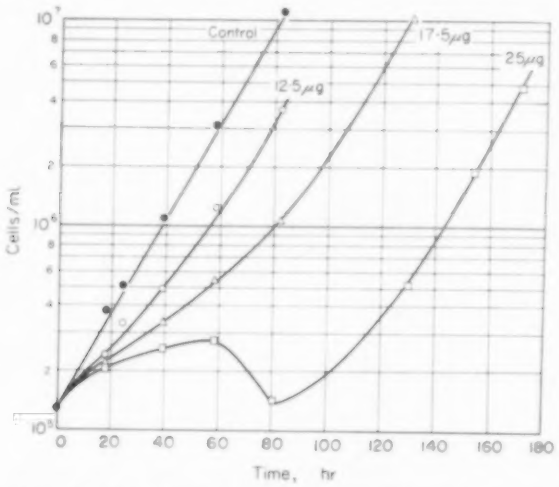

FIG. 2. Growth of leukaemia cells in tissue culture containing different quantities of dimethyl myleran ($CH_3.SO_2.O.CH(CH_3).(CH_2)_2.CH(CH_3).O.SO_2.CH_3$). The concentrations are in μg/ml.

only, so that there is almost no difference between the treated and the control cultures after 18 hr. Only on the second day can the effect of the cytotoxic agent be observed in that no further increase in cell numbers occurs and grossly abnormal cells are seen. With higher doses of dimethyl myleran the mitotic rate is reduced immediately, but the cells still divide at least once and abnormal cells are only seen after the initial population has doubled.

Following this initial period of growth, cell degeneration occurs and fragmentation is obvious after 40 hr. The actual cell count is then approximately constant (see Fig. 2), but after this a new population of cells makes its appearance and cell numbers increase at the same rate as with the controls. This, apparently stationary, phase also occurs after treatment with HN2, but in this case it sets in immediately after treatment. The exact time sequence depends on the dose of cytotoxic agent that is used.

Dose–response relationship

The new population of cells arises, we assume, from those cells that were not damaged by treatment with the alkylating agents. The number of these so-called survivors can be obtained by extrapolating the growth curves of the new population to zero-time (see dotted lines in Fig. 1).

This method of determining the fraction of the original population which has been damaged in such a way that it does not give rise to fully viable daughter cells is open to criticism on the grounds that the drugs may induce a temporary mitotic arrest from which the cells recover completely. If this occurs then the percentage of cells that have lost their capacity to multiply indefinitely, would be less than the value

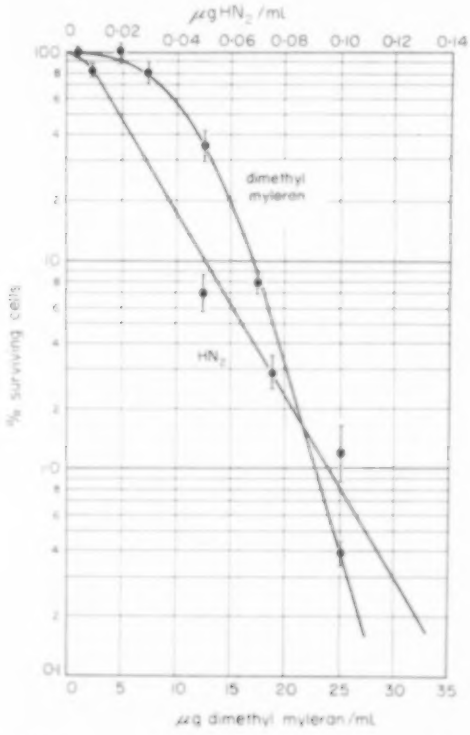

FIG. 3. Relationship between percentage of cells surviving (i.e. percentage of cells in original inoculum that retained ability to divide normally) and concentration of alkylating agents added to the tissue culture medium.

calculated by the extrapolation technique. We feel, however, that this objection is not valid, since small doses of dimethyl myleran cause almost no immediate reduction in the rate of cell division, although more than 50 per cent of the cells can be seen to be abnormal at 48 hr after treatment. These lymphoblasts would appear to be relatively resistant to the induction of mitotic delay which seems to occur only after sufficient damage has been done to render the cell incapable of repeated cell division.

Fig. 3 shows the dose–response curve determined by the extrapolation technique. Again, there is a qualitative difference between the two alkylating agents. The curve

of log survivors against dose is almost linear for HN2 and there is only a barely perceptible threshold. If one cared to express this curve according to "target theory" it would be 1·3 hit. However, this close approximation to a single hit inactivation curve cannot be interpreted along the line that one target molecule has to be inactivated.[19]

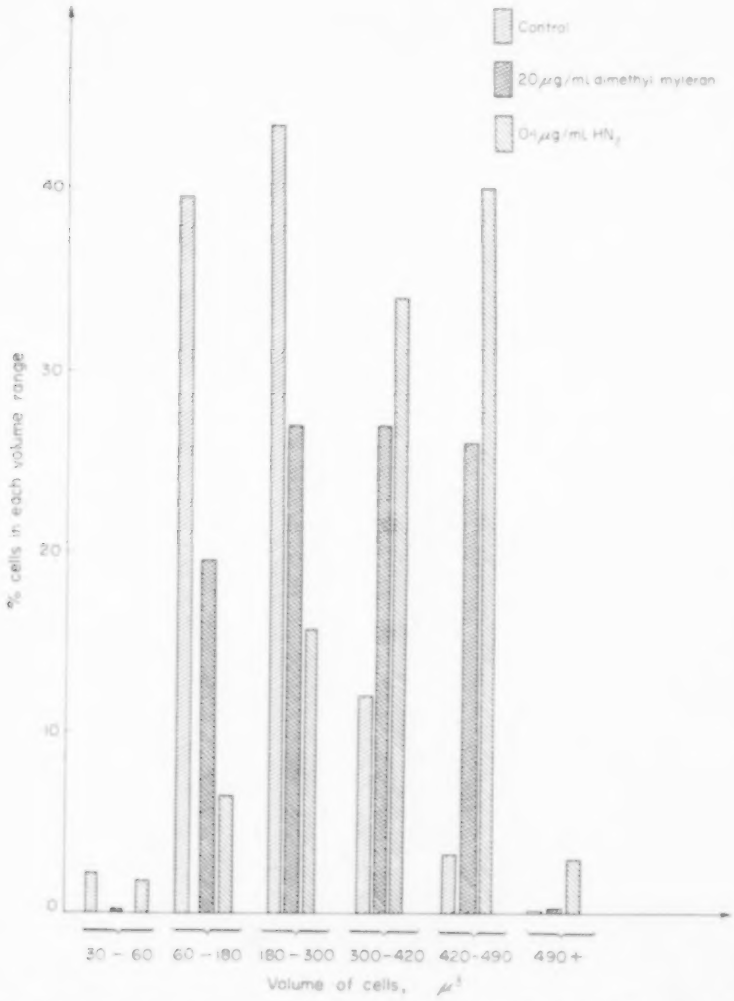

FIG. 4. Size distribution of leukaemia cells following treatment with dimethyl myleran and HN2.

From a practical point of view this type of dose–response curve indicates that the dose of HN2 needed to eradicate leukaemia *in vivo* (i.e. the dose needed to prevent every cell from proliferating) will depend on the number of cells present in the animal.

With dimethyl myleran the graph of the logarithm of survivors against dose is far from linear and shows a pronounced threshold (in the terminology of the target theory it would be 3·5 hit). In this respect dimethyl myleran shows a more typical

pharmacological response. Inspection of Fig. 3 shows that a value for the relative effectiveness of the two agents on a weight-basis depends on the level of cell "killing" at which the comparison is made. The difference in lethality is much greater at higher levels of survival.

Appearance of alkylated cells

Cells treated with dimethyl myleran appear quite normal under the microscope until they have divided at least once, when some of them turn into giant cells similar to those described by Puck and Marcus[11] in Hela cell cultures that have been irradiated with X-rays. Both the nucleus and cytoplasm increase in diameter to more than double to give cells having ten to fifty times the volume of the untreated. However,

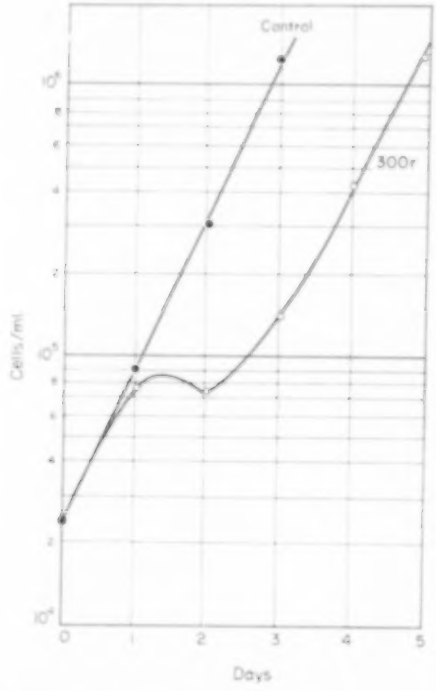

FIG. 5. Effect of a dose of 300 r of X-rays (220 kpV, 270 r/min) on the growth of a suspension of leukaemia cells. (To ensure that the suspension was not depleted of oxygen, an inoculum from a concentrated cell suspension was added to fresh medium immediately before irradiation.)

by no means all the cells that are "killed" turn into giants; 20 μg/ml gives rise to less than 15 per cent giants even though 95 per cent of the cells are killed. These giant cells are very fragile and degenerate after a few days.

After treatment with HN2, the cells already show abnormal features after 24 hr—in particular, the appearance of dark granules. The cell size increases after division has been stopped but the giant cells typical of dimethyl myleran are not seen. The HN2 cells degenerate more quickly and at all times the cells look quite different from those treated with dimethyl myleran. The immediate cessation of cell division by

HN2 would also not appear to be due to interference with DNA synthesis as this continues apparently normally for almost a day. Detailed biochemical investigations are now being made on the cells treated with these two alkylating agents.

Using the "Coulter Counter", changes in the size distribution following treatment can be seen (see Fig. 4). After 18 hr the volume of the HN2 treated cells is more than doubled and only 20 per cent of the cells are within the size range of the control cells. At later periods, fragments appear from degenerating cells but the size of the cells does not increase further.

With dimethyl myleran, a change in the size distribution is seen after 18 hr though to a much less extent. After 40 hr the distribution moves further to the right, but it does not peak at a high value as with HN2. A small tail of giant cells which are noticeable under the microscope become detectable in the "Coulter Counter" after 40 hr.

DISCUSSION

In its effect on the rate of cell division for these leukaemia cells dimethyl myleran is truly radiomimetic. With doses of X-rays up to 300 r—when 90 per cent of the cells are prevented from indefinite multiplication—growth in the culture only stops after the population has doubled (see Fig. 5).[12] The shape of the dose–response curve and the appearance of the pathological cells under the microscope is also extremely similar for dimethyl myleran and for X-rays.[11] The changes produced by HN2, on the other hand, are entirely different. It causes immediate cessation of cell division only seen after more than 2000 r, but even then the cells differ in appearance from those exposed to radiation, which even at these doses leads to the formation of giants.

After treatment with a nitrogen mustard, changes have been detected in the DNA of a variety of cells[2, 6, 13] which suggest that different DNA molecules have been joined together (cross-linked) by alkylation through the two centres present in the mustard molecule. It seems plausible that cross-linking of this kind will prevent cell division and that it is this reaction which is responsible for the immediate inhibition of growth. Dimethyl myleran has also been shown to cross-link the DNA within the nucleus,[13] but only at extremely high doses. For this reason Alexander and Lett[6] were led to suggest that at the chemical level the mode of action of the myleran type of compounds might be different from that of the mustards. Perhaps alkylation of a site other than the DNA is responsible and acid groups in internal cell barriers have been proposed.[6]

It is not clear whether the delay that is seen after treatment with dimethyl myleran is due to the fact that a cell division is necessary before the injury manifests itself e.g. by chromosome deletion effects[14] or whether metabolism alone is sufficient to develop the injury.

Acknowledgements—This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital), from the Medical Research Council, the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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Biochemical Pharmacology, 1961, Vol. 5, pp. 283–286. Pergamon Press Ltd., Printed in Great Britain.

ANTICONVULSANT PROPERTIES OF DIACETYLMONOXIME (DAM)

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(*Received* 23 *June* 1960)

Abstract—Both diacetylmonoxime and hydroxylamine protect mice against the lethal effects of pentylenetetrazol (Metrazol); neither compound protects against the lethal effects of thiosemicarbazide. Hydroxylamine elevates the levels of γ-aminobutyric acid (GABA) in rats, while diacetylmonoxime does not. Neither compound elevates brain GABA levels in mice at anticonvulsant doses. The causal relationship between brain GABA levels and seizures is questioned.

RECENTLY two reports have appeared which demonstrate the ability of hydroxylamine (NH_2OH) to elevate the level of γ-aminobutyric acid (GABA) in rat brain[1] and the anticonvulsant properties of NH_2OH and elevation of cerebral GABA in cats.[2] The authors postulated that the anticonvulsant properties of NH_2OH are due to its ability to inhibit selectively vitamin B_6-dependent GABA-α-ketoglutaric acid transaminase (the enzyme presumed to be responsible for the metabolic utilization of GABA in brain), thereby increasing the level of brain GABA. The toxic properties of NH_2OH [1, 2] make it improbable that this compound could be used therapeutically for treatment of convulsant disorders. In view of these facts, attention was focused on several analogs of NH_2OH developed as cholinesterase reactivators. Diacetylmonoxime (DAM) was chosen from among these compounds because it is known to penetrate the blood brain barrier.[3] The following exchange reaction, well known to organic chemists,[4]

$$\begin{matrix} O & & N{-}OH & & N{-}OH & & O \\ \| & & \| & & \| & & \| \\ R{-}CH & + & R_1{-}C{-}R_2 & \rightarrow & R{-}CH & + & R_1{-}C{-}R_2 \end{matrix}$$

indicates that DAM might inactivate vitamin B_6 in a manner similar to NH_2OH. In addition, the toxicity of DAM in man has been studied by Jager and Stagg,[5] who reported that 30 mg of DAM per kg, given intravenously, produces transient coma. This report presents data obtained in preliminary experiments testing the effectiveness of DAM against convulsions in mice and rats caused by pentylenetetrazol (Metrazol) and thiosemicarbazide (TSC). Data on brain GABA levels, after treatment with DAM and NH_2OH, are also presented.

METHODS AND MATERIALS

White mice of the Swiss Webster strain weighing 23–28 g and Wistar rats weighing 150–280 g were used. DAM and NH_2OH hydrochlorides were used as obtained from Distillation Products, Inc. DAM was dissolved in distilled water and adjusted to pH 7;

NH_2OH was dissolved in distilled water and adjusted to pH 7·4. Metrazol solution (0·1 g/ml), as obtained from Knoll Laboratories, Orange, N.J., was diluted with saline. TSC, obtained from Aldrich Chemical Co., Milwaukee, Wis., was dissolved in saline.

Estimation of brain GABA was carried out by excising brains at various times after administration of DAM, NH_2OH or saline. The weighed brains were extracted with hot 95% ethanol and the extract was evaporated to dryness. The resulting material was taken up in a known volume of distilled water and an aliquot spotted for chromatography. One-dimensional chromatography was carried out in a phenol-water solvent.[6] A solution of 0·4% ninhydrin, 10% isopropanol and 5% collidine in water was used for color development.[7] Color was extracted from the paper with hot water and the optical density determined at 570 mμ. Recovery (94–108 per cent) of known amounts of GABA added to brain was achieved routinely.

RESULTS

Mice

DAM (400 mg/kg)* given intraperitoneally protected mice from the lethal effects of Metrazol (Table 1). This dose of DAM produced transient ataxia in most mice

TABLE 1. PROTECTION OF MICE AND RATS FROM CONVULSIVE AGENTS

Protective agent (mg/kg)	Species	Delay min	Convulsant (mg/kg)	Fraction surviving
Saline	Mouse	15	Metrazol (100)	2/31
DAM(400)			Metrazol (100)	18/19*
Saline			TSC(20)	0/10
DAM(400)			TSC(20)	0/10
Saline		60	Metrazol (100)	3/32
NH_2OH(32)			Metrazol(100)	16/32*
Saline			TSC(20)	0/10
NH_2OH(32)			TSC(20)	0/10
Saline	Rats	15	Metrazol(150)	0/10
DAM(300)			Metrazol(150)	8/10*
Saline		60	Metrazol(200)	0/10
DAM(300)			Metrazol(200)	6/10*
Saline			Metrazol(200)	0/8
NH_2OH(32)			Metrazol(200)	1/8
Saline		90	Metrazol(100)	3/10
NH_2OH(32)			Metrazol(100)	7/8*

Doses of DAM and NH_2OH were calculated as free base. DAM and NH_2OH were given intraperitoneally while Metrazol was given subcutaneously. "Delay" represents the time-interval between the doses of the protective agent and the convulsant. The fraction surviving was determined after 24 hr for mice and after one hour for rats. All saline-treated animals that died after injection with Metrazol did so within 30 min.

* Significantly different from control at $P = 0.05$ level (Chi square).

immediately after injection; however, all mice appeared to recover and showed normal behavior by the time Metrazol was administered 15 min later. Metrazol produced only slight tremors and a few minor clonic convulsions in DAM-treated animals, whereas all mice given saline had repetitive clonic convulsions and usually

* Higher doses of DAM (800 mg/kg) produced a comatose state which lasted about one hour; all mice tested fully recovered from such treatment.

expired within 5–20 min after injection with Metrazol. NH_2OH (32 mg/kg), given intraperitoneally, also protected mice from the lethal effects of Metrazol. Neither DAM nor NH_2OH protected mice from the convulsions and lethal effects of TSC (Table 1).

No significant difference in brain GABA levels between saline-treated or DAM-treated mice was noted in animals sacrificed 30 min, 1 hr, 3 hr and 6 hr, respectively, after injection (Table 2). In doses which gave protection from Metrazol convulsions, NH_2OH also failed to raise brain GABA levels in mice (Tables 1 and 2).

Rats

Both DAM and NH_2OH protected rats from the convulsions and lethal effects of Metrazol (Table 1). All rats pretreated with saline convulsed with the doses of Metrazol used. Only 2 of the 10 rats pretreated with DAM and 1 of the 8 pretreated with NH_2OH convulsed with similar doses of Metrazol. Baxter and Roberts[8] have also reported protection in rats given NH_2OH.

In contrast to data obtained in mice, anticonvulsant doses of NH_2OH elevated brain GABA levels in rats; at doses which gave equal or better protection, DAM had no such effect (Table 2).

TABLE 2. BRAIN GABA LEVELS

Protector (mg/kg)	Time hr after injection	Species (number)	Brain GABA* μmoles/g
Saline	0·5	Mice (8)	2·3 ± 0·055
DAM(400)	0·5	,,	2·2 ± 0·066
Saline	1	,,	2·2 ± 0·21
DAM(400)	1	,,	2·2 ± 0·14
Saline	3	(4)	2·1 ± 0·20
DAM(400)	3	,,	2·0 ± 0·22
Saline	6	,,	2·0 ± 0·26
DAM(400)	6	,,	2·2 ± 0·24
Saline	1	(12)	2·2 ± 0·16
NH_2OH(32)	1	,,	2·2 ± 0·12
Saline	1	Rats (5)	2·3 ± 0·14
DAM(300)	1	,,	2·2 ± 0·08
Saline	1·5	(4)	1·8 ± 0·03
NH_2OH(35)	1·5	,,	2·9 ± 0·18†

Dosage and routes of administration were the same as those given in Table 1.
* Mean value ± s.e.
† Significantly different from control at $P = 0·05$ level (t-test).

DISCUSSION

DAM appears to have much in common with NH_2OH. Both compounds are carbonyl trapping agents; both protect mice against Metrazol convulsions without elevating brain GABA levels; and neither compound is capable of protecting mice from the lethal effects of TSC. These findings are consistent with the hypothesis that the two drugs produce their protective effects through similar mechanisms. Only NH_2OH is capable of elevating brain GABA levels in the rat, a fact which may have nothing to do with its protective effect.

The case for a causal relationship between brain GABA levels and seizures is currently being questioned. Results reported here, which show that NH_2OH and DAM do not elevate brain GABA levels in mice at doses which give good protection from lethal convulsions of Metrazol, indicate that GABA is not necessarily involved in the protective action of these drugs. In addition, recent results of Baxter and Roberts[9] show that thiosemicarbazide is capable of eliciting convulsions in rats at a time when brain GABA levels are still elevated from prior injection of NH_2OH. Further, Kessel[10] has reported that L-2,4-diaminobutyric acid elevates mouse brain GABA levels 2- to 3-fold, coincident with the occurrence of seizures. These findings have been confirmed by McKhann *et al.*[11]

DAM has been used in the treatment of anticholinesterase poisoning because of its ability to reactivate cholinesterase. It may also exert protective effects through an added anticonvulsant activity as well. The relationship between the anticonvulsant activity of DAM and its ability to reactivate cholinesterase is not clear.

Acknowledgement—The author expresses his gratitude to Ester L. Bugna for technical assistance.

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Biochemical Pharmacology, 1961, Vol. 5, pp. 287–304. Pergamon Press Ltd., Printed in Great Britain.

VERSUCHE ZUR ENTDECKUNG NEUER FUNGISTATIKA—II

NITRO-VERBINDUNGEN

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(*Received* 22 *August* 1960)

Abstract—The fungical action of 184 nitrogen compounds has been studied, both in 10 % beef serum containing dissolved mash–agar-culture medium, and in 3 % agar containing solid mash–agar-culture medium. The reaction mechanism under these conditions has been studied for those compounds which proved to be most active. Attempts were made to relate chemical structure to fungical activity, and to reaction mechanism, for this group of compounds.

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IN UNSERER früheren Mitteilung haben wir über die Ergebnisse berichtet, die wir bei der Untersuchung der in vitro ausgeübten fungistatischen Wirkung verschiedener substituierter Phenole und Phenol-äther, sowie beim Studieren ihres fungistatischen Wirkungsmechanismus erreicht hatten.[1]

In dieser Mitteilung beschäftigen wir uns mit der fungistatischen Wirkung der ein oder zwei Nitro-Gruppen enthaltenden, aliphatischen, aromatischen und heterozyklischen Verbindungen und auch mit ihrem biochemischen Wirkungsmechanismus.

Vor uns haben auch schon andere Verfasser die fungistatische Wirkung einzelner Nitro-Verbindungen studiert und eine fungistatische Wirkung von bedeutendem Grade bei einer Reihe von Verbindungen festgestellt: wie bei dem Trijod-nitro-äthylen und symm. Dijod-dinitro-äthylen,[2] dem Trichlor-nitro-methan,[3] den 4-Nitro-5-phenyl-cyklohexenen,[4] dem ω-Nitro-styrol und dessen verschiedenen Derivaten,[4–8] bei dem 1-*p*-Nitro-benzoyl-1-dichloracetylamino-2-hydroxy-äthan,[9] dem *o*-Nitro-benzaldehyd,[3] dem N-(*p*-Chlor-phenyl)-*o*-nitro-benzamid und dessen N-Methyl-Derivat,[10] bei den Mono- und Polyhalogen-Derivaten des Nitro-benzols,[11, 12] den substituierten und unsubstituierten Dinitro-benzolen,[12, 13] bei zahlreichen Nitro- und Dinitro-phenol-Derivaten,[3, 12, 14–20] Nitro-anilinen,[12] den 2-substituierten Derivaten des 5-Nitro-furans und 5-Nitro-thiophens[21–23] und endlich bei dem 2 : 5-Dichlor-3 : 4-dinitro-thiophen.[24]

Die Untersuchungen dieser Verfasser haben sich jedoch im allgemeinen auf eine grössere Anzahl von Verbindungen dieser Gruppe nicht ausgedehnt, so kann man daraus in Bezug auf die Abhängigkeit der fungistatischen Wirkung von der chemischen Struktur im Reiche der Nitro-Verbindungen noch keine Schlüsse ziehen.

Als Zielsetzung unserer Forschungen galt diesmal: einerseits, die *in vitro* ausgeübte fungistatische Wirkung einer verhältnismässig grossen Anzahl von Nitro-Verbindungen sowohl auf flüssigem als auch auf festem Nährboden festzustellen, miteinander zu vergleichen, die etwa bestehenden Zusammenhänge zwischen chemischer Struktur

und fungistatischer Wirkung in dieser Verbindungsreihe zu erkennen; andererseits aber: den Wirkungsmechanismus der im Laufe der Untersuchungen fungistatisch am intensivsten wirkenden Nitro-Verbindungen zu bestimmen und dadurch theoretische Anhaltspunkte zur Synthese weiterer wirksamer Verbindungen von ähnlichem Typus zu gewinnen.

Die Mehrheit der von uns untersuchten Nitro-Verbindungen haben wir gemäss den Methoden der chemischen Fachliteratur selbst hergestellt, der übrige, geringere Teil stand uns in der Form von Handelsprodukten in "pro analysi" Qualität zur Verfügung.

Die im Laufe der Untersuchung der fungistatischen Wirkung sowie des biochemischen Wirkungsmechanismus der Nitro-Verbindungen verfolgten Methoden waren mit denen, die in dem ersten Teil unserer Mitteilungsserie bereits beschrieben wurden,[1] ganz übereinstimmend, deshalb wollen wir hier auf diese nicht mehr eingehen. Es soll diesmal nur erwähnt werden, dass wir auch zu diesen Untersuchungen—wie auch für unsere früheren Forschungen bezüglich der fungistatischen Wirkung der Phenole-Derivate[1]—flüssigen Maische-Nährboden mit 10% Rinderserum-Gehalt sowie serumfreien festen Maische-Agar-Nährboden verwendet haben.

Zur Untersuchung des Wirkungsmechanismus haben wir ausserdem auch noch verschiedene Zusätze enthaltende flüssige, bzw. feste Maische-Agar-Nahrböden angewandt. Das pH der Kulturmedien betrug 6,0–6,2.

ERGEBNISSE

Die bei der Untersuchung der fungistatischen Wirkung der Nitro-Verbindungen erhaltenen Resultate sind in den Tabellen 1, 2 und 3 zusammengefasst.

UNTERSUCHUNGEN ÜBER DEN WIRKUNGSMECHANISMUS DER NITRO-VERBINDUNGEN

Beim Überblick der bei der Untersuchung der fungistatischen Wirkung der Nitro-Verbindungen erhaltenen Ergebnisse kann man feststellen, dass die am intensivsten wirkenden Verbindungen dieser Gruppe folgende strukturelle Eigenschaften aufweisen:

(1) Zu demselben Kohlenstoff-Atom, woran die Nitro-Gruppe gebunden ist, schliessen sich auch noch ein oder mehrere Halogenatome an (z.B. in den Verbindungen von Nr. F/267, 268, 273, 275, 283, 284).

(2) Die Nitro-Gruppe schliesst sich an irgendein Kohlenstoff-Atom doppelter Bindung eines ungesättigten araliphatischen Kohlenwasserstoffes an (z.B. in den Verbindungen von Nr. F/277, 281 und 282).

(3) In einem Benzol-Ring sind zwei Nitro-Gruppen in Verhältnis zueinander in Meta-Stellung anwesend (z.B. in den Verbindungen von Nr. F/290, 291, 292, 294, 315).

(4) Eine Nitro-Gruppe ist in den Benzyl-halogeniden in Verhältnis zur Gruppe $—CH_2—X$ in *Ortho*-Stellung anwesend (wie z.B. in den Verbindungen von Nr. F/316 und F/317).

(5) In Phenol-Derivaten sind in der im Verhältnis zu dem phenolischen Hydroxyl-Radikal gestellten *o*-, *m*- oder *p*-Stellung ein bzw. zwei Nitro-Gruppen und dabei vielleicht noch gelegentlich zugleich ein oder mehrere Alkyl-Substituenten oder Halogenatome anwesend (z.B. in den Verbindungen von Nr. F/325, 326, 328, 334, 335, 336, 337, 349, 351, 352, 356).

TABELLE I. DIE FUNGISTATISCHE WIRKUNG VON NITRO-VERBINDUNGEN AUF FLÜSSIGEM SERUM-MAISCHE NÄHRBODEN

Nr.	Verbindungen	*Penicillium simplicissimum*	*Aspergillus niger*	*Trichothecium roseum*	*Candida albicans*	*Achorion quinckeanum*	*Trichophyton gypseum*	*Eidermophyton* Kaufman-Wolff
	I. Aliphatische Nitro-Verbindungen							
F/264	Trichlor-nitro-methan (=Chlorpikrin)	5000	(5000)	10 000	5000	10 000	10 000	10 000
F/265	Tetranitro-methan	—	—	—	—	5000	5000	5000
F/266	2-Nitro-2-chlor-propan	10 000	5000	10 000	5000	10 000	10 000	25 000
F/267	1-Nitro-1:1-dibrom-aethan	50 000	25 000	50 000	25 000	50 000	50 000	50 000
F/268	1-Nitro-1:1-dibrom-propan	10 000	10 000	10 000	10 000	10 000	10 000	10 000
F/269	2-Nitro-2-brom-*n*-pentan	10 000	5000	25 000	10 000	25 000	25 000	25 000
F/270	3-Nitro-3-brom-*n*-pentan	10 000	5000	10 000	5000	25 000	25 000	25 000
F/271	2-Nitro-2-brom-*n*-heptan	5000	—	5000	5000	10 000	10 000	10 000
F/272	1-Nitro-1-brom-cyklopentan	10 000	10 000	10 000	10 000	10 000	10 000	10 000
F/273	1-Nitro-1-brom-cyklohexan	100 000	25 000	100 000	25 000	100 000	100 000	100 000
F/274	1-Nitro-1-brom-2-*i*-propyl-5-methyl-cyklohexan	5000	—	25 000	(5000)	10 000	10 000	10 000
F/275	1-Nitro-1-brom-2-methyl-2,5-(endo-dimethyl-methylen)-cyklohexan	50 000	10 000	50 000	25 000	50 000	100 000	100 000
	II. Araliphatische Nitro-verbindungen							
F/277	1-Phenyl-2-nitro-aethylen (=ω-nitro-styrol)	10 000	10 000	10 000	10 000	25 000	25 000	25 000
F/278	1-(2′-Hydroxy-phenyl)-2-nitro-aethylen	—	—	5000	—	10 000	10 000	10 000
F/279	1-(3′-Nitro-phenyl)-2-nitro-aethylen	—	—	5000	(5000)	5000	5000	5000
F/280	1-(4′-Dimethylamino-phenyl)-2-nitro-aethylen sulphat	—	—	—	—	5000	5000	5000
F/281	1-(2′-Furyl)-2-nitro-aethylen	5000	—	5000	—	10 000	10 000	10 000
F/282	1-(2′-Furyl)-2-methyl-2-nitro-aethylen	5000	—	5000	—	5000	5000	5000
F/283	1-Phenyl-1-nitro-1-brom-aethan	10 000	10 000	25 000	10 000	25 000	25 000	25 000
F/284	1-Phenyl-2-nitro-1:2-dibrom-aethan	50 000	25 000	50 000	25 000	100 000	100 000	100 000
	III. Aromatische Nitro-Kohlenwasserstoffe							
F/286	2-Nitro-toluol	(5000)	—	5000	—	10 000	10 000	10 000
F/287	3-Nitro-toluol	(5000)	—	5000	—	10 000	10 000	10 000
F/288	4-Nitro-toluol	5000	—	5000	—	10 000	10 000	10 000
F/289	Nitro-xylol (Gemisch von Isomeren)	10 000	5000	10 000	5000	10 000	10 000	10 000
F/290	1:3-Dinitro-benzol	25 000	25 000	10 000	(5000)	10 000	10 000	10 000
F/291	2:4-Dinitro-toluol	5000	5000	5000	(5000)	5000	5000	5000
F/292	3:5-Dinitro-toluol	5000	(5000)	5000	—	5000	5000	5000

Tabelle 1—*fortges.*

Nr.	Verbindungen	*Penicillium simplissimum*	*Aspergillus niger*	*Trichothecium roseum*	*Candida albicans*	*Achorion quinckeanum*	*Trichophyton gypseum*	*Epidermophyton* Kaufman-Wolff
F/293	Dinitro-xylol (Gemisch von Isomeren)	5000	5000	10 000	5000	10 000	10 000	10 000
F/294	2:4:6-Trinitro-toluol	5000	—	10 000	(5000)	10 000	10 000	10 000
F/295	4-Nitro-diphenyl	—	—	5000	—	5000	5000	5000
F/297	1-Nitro-naphtalin	5000	5000	10 000	5000	10 000	10000	10 000
F/298	1-Nitro-2-methyl-naphtalin	—	—	—	—	10 000	10 000	10 000
F/299	1-Nitro-4-methyl-naphtalin	—	—	10 000	—	10 000	10 000	10 000
F/300	9-Nitro-phenanthren	—	—	10 000	—	10 000	10 000	10 000
F/306	1:5-Dinitro-naphtalin	—	—	—	—	10 000	10 000	10 000
	IV. Halogen-substituierte aromatische Nitro-Kohlenwasserstoffe							
F/307	2-Nitro-chlor-benzol	5000	—	5000	—	10 000	10 000	10 000
F/308	4-Nitro-chlor-benzol	5000	(5000)	10 000	—	10 000	10 000	10 000
F/309	3-Nitro-jod-benzol	5000	(5000)	5000	—	10 000	10 000	10 000
F/310	4-Nitro-jod-benzol	5000	—	10 000	—	10 000	10 000	10 000
F/311	2-Nitro-4-chlor-toluol	5000	—	10 000	—	10 000	10 000	10 000
F/312	2:5-Dichlor-nitro-benzol	5000	5000	10 000	—	10 000	10 000	10 000
F/313	3:4-Dichlor-nitro-benzol	5000	(5000)	5000	—	10 000	10 000	10 000
F/314	3:5-Dibrom-nitro-benzol	25 000	(5000)	25 000	5000	25 000	25 000	25 000
F/315	2:4-Dinitro-chlor-benzol	100 000	50 000	100 000	25 000	100 000	100 000	100 000
F/316	2-Nitro-benzyl-chlorid	10 000	10 000	25 000	10 000	50 000	50 000	50 000
F/317	2-Nitro-benzyl-jodid	25 000	25 000	50 000	25 000	50 000	50 000	50 000
F/318	1-Nitro-4-chlor-naphtalin	10 000	5000	10 000	10 000	25 000	25 000	25 000
F/319	4-Nitro-9-brom-phenanthren	—	—	—	—	10 000	10 000	10 000
	V. Mononitro-phenole							
F/324	2-Nitro-phenol	5000	5000	10 000	5000	10 000	10 000	10 000
F/325	3-Nitro-phenol	5000	—	10 000	5000	10 000	10 000	10 000
F/326	4-Nitro-phenol	5000	(5000)	10 000	(5000)	10 000	10 000	10 000
F/327	2-Nitro-4-methyl-phenol	(5000)	(5000)	(5000)	—	(5000)	(5000)	(5000)
F/328	2-Nitro-4:6-dimethyl-phenol	(5000)	—	5000	—	10 000	10 000	10 000
F/329	4-Nitro-2-*i*-propyl-5-methyl-phenol	—	—	5000	—	5000	5000	10 000
F/330	3-Nitro-4-hydroxy-diphenyl	—	—	10 000	5000	25 000	25 000	25 000
F/331	2-Nitro-4-benzyloxy-phenol	—	—	—	(5000)	5000	5000	5000
F/332	2-Nitro-4-*n*-hexyloxy-phenol	—	—	—	(5000)	10 000	10 000	10 000
F/333	2-Nitro-4-*n*-octyloxy-phenol	—	—	—	—	(5000)	(5000)	(5000)
F/334	2-Nitro-4-propionyl-phenol	(5000)	—	5000	—	10 000	10 000	10 000

TABELLE 1—*fortges.*

Nr.	Verbindungen	*Penicillium simplicissimum*	*Aspergillus niger*	*Trichothecium roseum*	*Candida albicans*	*Achorion quinckeanum*	*Trichophyton gypseum*	*Epidermophyton* Kaufman-Wolff
F/335	2-Nitro-4-chlor-phenol	10 000	10 000	10 000	5000	10 000	10 000	10 000
F/336	2-Nitro-4:6-dibrom-phenol	10 000	5000	10 000	5000	10 000	10 000	10 000
F/337	3-Nitro-4:6-dibrom-phenol	10 000	5000	10 000	5000	10 000	10 000	10 000
F/338	4-Nitro-2:6-dibrom-phenol	5000	5000	5000	—	5000	5000	5000
F/339	2-Amino-4-nitro-phenol	—	—	—	—	5000	5000	5000
F/340	2-Amino-4-nitro-6-methyl-phenol	—	—	5000	—	5000	5000	5000
F/344	3-Nitro-4-hydroxy-benzoesäure-*n*-propyl-ester	—	—	5000	—	10 000	10 000	10 000
F/345	3-Methyl-4-hydroxy-5-nitro-azobenzol	—	—	—	—	5000	5000	5000
F/346	3-Chlor-4-hydroxy-5-nitro-azobenzol	—	—	—	—	10 000	10 000	10 000
	VI. Dinitro-phenole							
F/348	2:4-Dinitro-phenol	(5000)	(5000)	(5000)	—	5000	5000	5000
F/349	2:5-Dinitro-phenol	10 000	10 000	10 000	5000	10 000	10 000	10 000
F/351	2:4-Dinitro-6-methyl-phenol	10 000	(5000)	5000	(5000)	5000	5000	10 000
F/352	2:4-Dinitro-5-methyl-phenol	5000	(5000)	5000	(5000)	10 000	10 000	10 000
F/353	2:6-Dinitro-4-methyl-phenol	(5000)	(5000)	(5000)	—	5000	5000	5000
F/354	2:6-Dinitro-3,4-dimethyl-phenol	(5000)	(5000)	5000	—	10 000	10 000	10 000
F/355	3:5-Dinitro-4-hydroxy-diphenyl	—	—	—	—	10 000	5000	5000
F/356	2:4-Dinitro-6-chlor-phenol	5000	5000	5000	5000	10 000	5000	10 000
F/357	2:6-Dinitro-4-chlor-phenol	5000	5000	5000	—	5000	5000	5000
	VII. Nitro-naphtole							
F/364	2-Amino-4-nitro-1-naphtol	—	—	—	—	(5000)	(5000)	5000
F/365	2,4-Dinitro-1-naphtol	—	—	(5000)	(5000)	5000	5000	5000
	VIII. Nitro-Derivate von Phenol-aethern							
F/369	2-Nitro-4-methyl-1-aethoxy-benzol	(5000)	—	5000	—	10 000	10 000	10 000
F/370	2-Nitro-4-methyl-1-allyloxy-benzol	(5000)	—	5000	—	10 000	10 000	10 000
F/371	2-Nitro-4-methyl-1-*n*-hexyloxy-benzol	—	—	—	—	5000	5000	5000
F/372	2-Nitro-4-methyl-1-benzyloxy-benzol	—	—	—	—	10 000	10 000	10 000
F/373	4-Nitro-2-i-propyl-5-methyl-1-aethoxy-benzol	—	—	—	—	5000	5000	5000
F/377	3-Nitro-4-aethoxy-diphenyl	—	—	—	—	10 000	10 000	10 000
F/379	2-Nitro-4-chlor-1-aethoxy-benzol	5000	—	5000	—	5000	5000	5000
F/380	2-Nitro-4-chlor-1-allyloxy-benzol	(5000)	—	(5000)	—	5000	5000	5000

TABELLE 1—*fortges.*

Nr.	Verbindungen	*Penicillium simplicissimum*	*Aspergillus niger*	*Trichothecium roseum*	*Candida albicans*	*Achorion quinckeanum*	*Trichyphyton gypseum*	*Epidermophyton* Kauffman-Wolff
F/381	2-Nitro-4-chlor-1-*n*-hexyloxy-benzol	—	—	—	—	5000	5000	5000
F/387	1-Nitro-2-allyloxy-naphtalin	—	—	—	—	10 000	10 000	10 000
	IX. Nitro-aniline und ihre Derivate							
F/399	2-Nitro-anilin	—	—	5000	—	5000	5000	5000
F/400	3-Nitro-anilin	5000	5000	5000	—	5000	5000	5000
F/401	4-Nitro-anilin	—	—	(5000)	—	5000	(5000)	5000
F/402	2-Nitro-4-methyl-anilin	10 000	—	5000	5000	10 000	10 000	10 000
F/403	2-Nitro-4-aethoxy-anilin	10 000	—	5000	5000	10 000	5000	10 000
F/404	2-Nitro-4-chlor-anilin	10 000	(5000)	10 000	5000	10 000	10 000	10 000
F/405	4-Nitro-2-methyl-anilin	—	—	(5000)	—	(5000)	(5000)	5000
F/406	4-Nitro-3-methyl-anilin	—	—	(5000)	—	(5000)	(5000)	5000
F/407	2 : 4-Dibrom-5-nitro-anilin	5000	5000	10 000	—	10 000	10 000	10 000
F/408	2 : 6-Dibrom-4-nitro-anilin	5000	5000	10 000	—	10 000	10 000	10 000
F/409	2 : 6-Dijod-4-nitro-anilin	5000	5000	10 000	—	25 000	25 000	25 000
F/420	1-Amino-4-nitro-naphtalin	(5000)	—	5000	—	10 000	10 000	10 000
F/422	2 : 4-Dinitro-anilin	—	—	—	—	5000	5000	5000
F/423	2 : 4-Dinitro-diaethylanilin	—	—	—	—	5000	5000	5000
F/424	2 : 4-Dinitro-di-(*n*-butyl)-anilin	—	—	—	—	5000	(5000)	5000
F/425	2 : 4-Dinitro-cyklohexyl-anilin	—	—	—	—	5000	5000	5000
F/427	N-(2 : 4-Dinitro-phenyl)-piperidin	—	—	—	—	10 000	10 000	10 000
F/428	N : N′-Di-(2 : 4-dinitro-phenyl)-piperazin	—	—	5000	—	5000	5000	5000
F/429	1 : 6-Di-(2′ : 4′-dinitro-phenyl-amino)-hexan	—	—	—	—	5000	5000	5000
	X. Heterozyklische Nitro-Verbindungen							
F/400	6-Nitro-1 : 2 : 3 : 4-tetrahydro-carbazol	—	—	—	—	(5000)	(5000)	(5000)
F/441	5-Nitro-chinolin	—	—	—	—	5000	5000	5000
F/442	2-Methyl-6-nitro-chinolin	—	—	5000	—	5000	5000	5000
F/443	2-Methyl-8-nitro-chinolin	—	—	5000	—	5000	5000	10 000
F/444	2-Methyl-6-aethoxy-8-nitro-chinolin	(5000)	—	5000	—	5000	5000	5000

Die in der Tabelle stehenden Ziffern bezeichnen die Reziprokwerte jener Verdünnung der einzelnen Verbindungen, welche zur vollkommenen *Entwicklungshemmung* führen. — = Die untersuchte Verbindung übt auch in der angewandten niedrigsten Verdünnung—in 1 : 5000—keine fungistatische Wirkung aus. (5000) = Die untersuchte Verbindung kann auch in der angewandten niedrigsten Verdünnung—in 1 : 5000—nur eine teilweise Entwicklungshemmung des Pilzstammes herbeiführen.

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TABELLE 2. NITRO-VERBINDUNGEN, WELCHE IN FLÜSSIGEM SERUM-MAISCHE NÄHRBODEN AUF JEDE UNTERSUCHTE PILZSTÄMME VOLLSTÄNDIG UNWIRKSAM SIND

F/261	Nitro-methan	F/375	4-Nitro-2-*i*-propyl-5-methyl-1-*n*-octyloxy-benzol	F/426	2 : 4-Dinitro-diphenylamin
F/262	Nitro-aethan	F/376	4-Nitro-2-*i*-propyl-5-methyl-1-benzyloxy-benzol	F/430	1-(4′-Nitro-phenyl-amino)-2 : 5-dimethyl-pyrrol
F/263	1-Nitro-propan	F/378	3-Nitro-4-benzyloxy-diphenyl	F/431	1-(2′ : 4′-Dinitro-phenyl-amino)-2 : 5-dimethyl-pyrrol
F/276	Phenyl-nitro-methan	F/382	2-Nitro-4-chlor-1-*n*-octyloxy-benzol	F/432	N-(2 : 4-Dinitro-phenyl)-pyridinium-chlorid
F/285	Nitro-benzol	F/383	6-Nitro-2-*i*-propyl-5-methyl-4-brom-1-aethoxy-benzol	F/433	N-(2 : 4-Dinitro-phenyl)-chinolinium-chlorid
F/296	4 : 4′-Dinitro-diphenyl	F/384	2 : 4-Dinitro-4′-methyl-diphenyl-aether	F/434	N-(2-Nitro-benzyl)-pyridinium-chlorid
F/301	9-Nitro-anthrazen	F/385	2 : 4-Dinitro-2′-*i*-propyl-5′-methyl-diphenyl-aether	F/435	1-(2′ : 4′-Dinitro-phenyl)-3 : 5-dimethyl-pyrazol
F/302	2-Nitro-chrysen	F/386	1-Nitro-2-aethoxy-naphtalin	F/436	1-(4′-Nitro-phenyl)-3-methyl-4-nitro-pyrazolon-(5) (= Pikrolon-säure)
F/303	4-Nitro-acenaphten	F/388	1-Nitro-2-*n*-octyloxy-naphtalin	F/437	2-Acetylamino-5-nitro-thiazol
F/304	4-Nitro-fluoranthen	F/389	1-Nitro-2-*n*-dodecyloxy-naphtalin	F/437	2-Acetylamino-4-methyl-5-nitro-thiazol
F/305	3-Nitro-pyren	F/390	1-Nitro-2-benzyloxy-naphtalin	F/439	2-*n*-Butyrylamino-4-methyl-5-nitro-thiazol
F/320	9-Nitro-10-brom-anthrazen	F/391	4-Nitro-1-aethoxy-naphtalin		
F/321	8-Nitro-2-brom-chrysen	F/392	4-Nitro-1-*n*-octyloxy-naphtalin		
F/322	12-Nitro-4-brom-fluoranthen	F/393	4-Nitro-1-benzyloxy-naphtalin		
F/323	10-Nitro-3-brom-pyren	F/394	3-Nitro-benzoesäure		
F/341	2-Amino-6-nitro-4-methyl-phenol	F/395	4-Nitro-benzoesäure		
F/342	2-Amino-6-nitro-4-chlor-phenol	F/396	3, 5-Dinitro-benzoesäure		
F/343	3-Nitro-4-hydroxy-benzoesäure	F/397	4-Nitro-phenyl-essigsäure		
F/347	2-Nitro-phenol-4-sulphosäure-Kalium	F/398	4-Nitro-1-naphtyl-essigsäure		
F/350	2 : 6-Dinitro-phenol	F/410	4-Nitro-acetanilid		
F/358	2 : 4-Dinitro-6-amino-phenol	F/411	2-Nitro-4-methyl-acetanilid		
F/359	2-Hydroxy-3 : 5-dinitro-benzoesäure	F/412	2-Nitro-4-aethoxy-acetanilid		
F/360	2 : 6-Dinitro-resorzin	F/413	2-Nitro-4-chlor-acetanilid		
F/361	2 : 4 : 6-Trinitro-phenol (= Picrin-säure)	F/414	4-Nitro-2-methyl-acetanilid		
F/362	Phenol-purpursaure-Natrium	F/415	4-Nitro-3-methyl-acetanilid		
F/363	Dinitro-phenolphtalein	F/416	4-Nitro-dimethylanilin		
F/366	2 : 4-Dinitro-1-naphtol-7-sulphosäure-Natrium	F/417	4-Nitro-diaethylanilin		
F/367	Naphtol-purpursäure-Natrium	F/418	2-Nitro-4 : 4′-diamino-dpihenyl		
F/368	10-Nitro-9-anthron	F/419	4-Nitro-diphenylamin		
F/374	4-Nitro-2-*i*-propyl-5-methyl-1-allyloxy-benzol	F/421	1-Acetylamino-4-nitro-naphtalin		

TABELLE 3. DIE FUNGISTATISCHE WIRKUNG VON NITRO-VERBINDUNGEN AUF FESTEM MAISCHE-AGAR NÄHRBODEN

Nr.	Verbindungen	*Penicillium simplicissimum*	*Aspergillus niger*	*Trichothecium roseum*	*Candida albicans*	*Saccharomyces cerevisiae*
F/267	1-Nitro-1 : 1-dibrom-aethan	>90	>90	>90	>90	>90
F/268	1-Nitro-1 : 1-dibrom-propan	>90	>90	>90	>90	>90
F/277	1-Phenyl-2-nitro-aethylen (= ω-Nitro-styrol)	22	36	50	>90	63
F/281	1-(2'-Furyl)-2-nitro-aethylen	25	35	50	50	43
F/282	1-(2'-Furyl)2--methyl-2-nitro-aethylen	35	50	20	12	12
F/283	1-Phenyl-1-nitro-1-brom-aethan	10	12	12	17	25
F/284	1-Phenyl-2-nitro-1 : 2-dibrom-aethan	27	25	57	60	57
F/290	1 : 3-Dinitro-benzol	55	30	30	20	12
F/291	2 : 4-Dinitro-toluol	20	27	25	10	10
F/292	3 : 5-Dinitro-toluol	20	30	25	17	15
F/293	Dinitro-xylol (Gemisch von Isomeren)	15	17	20	10	10
F/294	2 : 4 : 6-Trinitro-toluol	27	30	25	22	20
F/315	2 : 4-Dinitro-chlor-benzol	40	30	27	30	30
F/316	2-Nitro-benzyl-chlorid	15	15	15	12	17
F/317	2-Nitro-benzyl-jodid	30	27	37	40	37
F/325	3-Nitro-phenol	28	25	18	—	—
F/326	4-Nitro-phenol	28	26	17	—	—
F/328	2-Nitro-4 : 6-dimethyl-phenol	20	33	17	12	12
F/329	4-Nitro-2-*i*-propyl-5-methyl-phenol	10	15	10	10	12
F/330	3-Nitro-4-hydroxy-diphenyl	10	15	15	20	12
F/334	2-Nitro-4-propionyl-phenol	20	22	36	25	18
F/335	2-Nitro-4-chlor-phenol	40	32	40	10	10
F/336	2-Nitro-4 : 6-dibrom-phenol	24	35	40	28	17
F/337	3-Nitro-4 : 6-dibrom-phenol	30	30	42	30	32
F/338	4-Nitro-2 : 6-dibrom-phenol	16	20	18	10	10
F/339	2-Amino-4-nitro-phenol	10	10	20	12	15
F/340	2-Amino-4-nitro-6-methyl-phenol	30	35	25	15	15
F/344	3-Nitro-4-hydroxy-benzoesäure-*n*-propyl-ester	20	17	20	20	10
F/346	3-Chlor-4-hydroxy-5-nitro-azobenzol	10	10	20	12	10
F/348	2 : 4-Dinitro-phenol	17	30	20	10	10
F/349	2 : 5-Dinitro-phenol	45	40	42	22	25
F/351	2 : 4-Dinitro-6-methyl-phenol	20	30	20	20	22
F/352	2 : 4-Dinitro-5-methyl-phenol	26	42	50	18	17
F/354	2 : 6-Dinitro-3 : 4-dimethyl-phenol	15	18	25	—	—
F/356	2 : 4-Dinitro-6-chlor-phenol	40	40	38	17	18

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TABELLE 3—*forgtes.*

Nr.	Verbindungen	*Penicillium simplicissimum*	*Aspergillus niger*	*Trichothecium roseum*	*Candida albicans*	*Saccharomyces cerevisiae*
F/364	2-Amino-4-nitro-1-naphtol	13	15	15	—	—
F/365	2 : 4-Dinitro-1-naphtol	18	22	20	—	—
F/402	2-Nitro-4-methyl-anilin	35	18	28	10	10
F/403	2-Nitro-4-aethoxy-anilin	35	30	32	10	—
F/404	2-Nitro-4-chlor-anilin	18	15	30	—	—
F/420	1-Amino-4-nitro-naphtalin	10	15	15	10	10
F/422	2 : 4-Dinitro-anilin	40	44	50	35	30
F/443	2-Methyl-8-nitro-chinolin	14	18	15	10	10
F/444	2-Methyl-6-aethoxy-8-nitro-chinolin	12	20	15	10	12

Die in der Tabelle stehenden Ziffern bezeichnen die Durchmesser der durch die einzelnen Verbindungen herbeiführten wachstumsgehemmten Gebiete in mm ausgedrückt.

— = Die Verbindung übt auf den angewandte Pilzstamm keine fungistatische Wirkung aus.

Jene von den untersuchten Nitro-Verbindungen der Tabelle 1 und 2, welche in der Tabelle 3 nicht aufgeführt sind, übten auf festem Maische-Agar Nährboden keine fungistatische Wirkung auf die zu den Versuchen verwendeten fünf Pilzstämme aus.

(6) In den *p*-substituierten Anilinen ist in der *o*-Stellung zum NH_2-Radikal eine NO_2-Gruppe enthalten (z.B. in den Verbindungen von Nr. F/402, 403 und 404).

Diejenige Verbindungen, die über die in Punkten (1), (2) und (4), angeführten Strukturelemente verfügen, sind infolge der elektrophylen Eigenschaft der in ihrem Molekül anwesenden Nitro-Gruppe in bedeutendem Masse reaktionsfähig, deshalb können sie mit verschiedenen organischen Radikalen, wie z.B. auch mit HS-Gruppen, sehr leicht reagieren.

So scheint die Voraussetzung auf der Hand zu liegen, dass diese Verbindungen ihre fungistatische Wirkung durch die Hemmung der Sulfhydryl-Enzyme ausüben, welche zum intermediären Stoffwechsel und zur Vermehrung der Pilz-Zellen unerlässlich notwendig sind. Zur tatsächlichen Unterstützung dieser Annahme haben wir die auf das *Penicillium simplicissimum* und *Saccharomyces cerevisiae* ausgeübte fungistatische Wirkung der bei unseren auf flüssigem Nährboden ausgeführten Untersuchungen besonders wirksam erwiesenen Nitro-Verbindungen auch auf solchem Maische-Agar-Nährboden untersucht, welcher 0,3% Na-Thioglycolat oder 0,3% Cystein-hydrochlorid enthielt (pH = 6,2). Die Methode, die wir bei diesen Untersuchungen auf festem Nährboden mit Cystein-hydrochlorid oder Na-Thioglycolat verfolgt haben, war dieselbe, wie diejenige, welche bei unseren Untersuchungen auf einfachem Maische-Agar ohne solche Zusätze angewandt wurde.

Indem die auf Cystein-hydrochlorid oder Na-Thioglycolat enthaltendem festem Nährboden ausgeübte fungistatische Wirkung der untersuchten Nitro-Verbindung *wesentlich* niedriger ist, als diese Wirkung ohne solchen Zusätze (d.h. auf normalem Maische-Agar) oder auf Cystein-hydrochlorid und Na-Thioglycolat enthaltendem festem Maische-Agar-Nährboden bleibt sie möglicherweise vollkommen wirkungslos, während sie zur gleichen Zeit auf normalem festem Nährboden fungistatisch wirksam sein kann, in dem Falle kann es für bestätigt gelten, dass die Nitro-Verbindung mit den Sulfhydryl-Gruppe enthaltenden Verbindungen unter den angewandten experimentellen Umständen auf irgendeine Weise reagieren kann. Dadurch wird unsere Voraussetzung *bedeutend unterstützt*—wenn auch nicht zweifellos bestätigt—dass die betreffende Verbindung mit einzelnen, zur Vermehrung der Pilz-Zellen unerlässlich notwendigen Sulfhydryl-Enzymen auch auf ähnliche Weise reagieren kann, wodurch sie jene inaktiviert; durch die Inaktivierung dieser Enzyme wird dann die Fungistase notwendigerweise herbeigeführt.

Diese Modell-Versuche können vielleicht zur Klärung der Frage beitragen, ob in dem fungistatischen Wirkungsmechanismus einer Verbindung die durch sie eventuell verursachte Inaktivierung der Sulfhydryl-Enzyme der Pilz-Zellen mitwirken kann oder nicht.

Die Verbindungen mit den unter (3), (5) und (6) angeführten Strukturelementen—mit Ausnahme des 2 : 4-Dinitro-chlor-benzols (F/315)—sind laut unserer heutigen Kenntnisse unfähig mit Sulfhydryl-Gruppen enthaltenden Verbindungen auf Grund eines Mechanismus von Substitutions- oder Additions-Charakter zu reagieren—in Gegensatz zu den angeführten Verbindungstypen unter 1, 2 und 3—es können höchstens einige unter diesen, abhängig von den Umständen in höherem oder geringerem Masse oxidierend auf sie einwirken, wobei ihre Sulfhydryl-Gruppe sich in eine Disulfid-Gruppe umwandelt:

$$R—NO_2 + 2\ HS—R' \rightarrow H_2O + R—NO + R—S—S—R'$$

Zur Erklärung der Frage, ob die auf die Sulfhydryl-Enzyme der Pilz-Zellen ausgeübte etwaige oxidierende Wirkung in dem Wirkungsmechanismus dieser Verbindungen mitwirken kann, haben wir auch die Beeinflussung deren fungistatischen Wirkung infolge Cystein-hydrochlorid und Na-Thioglycolat untersucht.

Für die Verbindungen mit den unter (5) angeführten Strukturelementen ist die phenolische Hydroxyl-Gruppe mehr kennzeichnend als die Nitro-Gruppe. Infolge der elektrophylen Eigenschaft der in ihren Molekülen anwesenden Nitro-Gruppen vermag nämlich das H-Atom ihres phenolischen Hydroxyl-Radikals in demselben Masse als H^+- Ion dissozieren, wie das der Halogen-Phenole. Sie können also vielmehr als Phenol-Derivate von bedeutendem Dissoziationsgrad (d.h. von niedrigem pK_a) betrachtet werden, als für substituierte Derivate des Nitro-benzols oder Dinitro-benzols gelten.

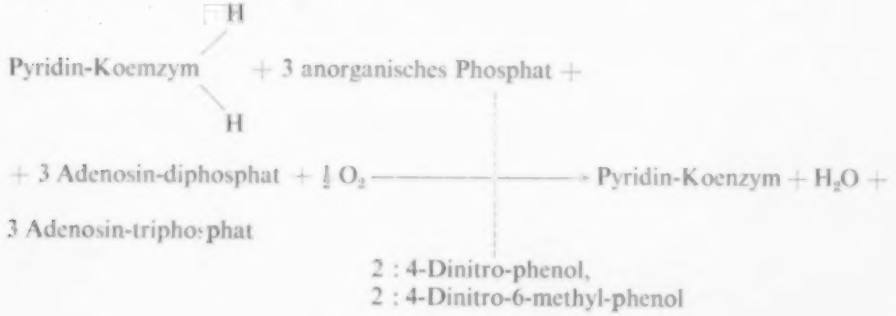
Es kann deshalb angenommen werden, dass sie ihre fungistatische Wirkung auf Grund desselben Mechanismus ausüben, wie die Nitro-Gruppen nicht enthaltenden Phenol-Derivate: d.h. durch ihre Bindung an die Eiweisse, Lipoide und Lipoproteide der Zellwand, bzw. der Zellhaut und auf dem Wege der dadurch verursachten Desorganisation (Vgl. Teil I dieser Mitteilungsserie).[1]

Zur Erklärung dieses Problems haben wir untersucht, ob die fungistatische Wirkung der wirksamsten gefundenen Nitro-phenol-Derivate so im festen wie auch im flüssigen Nährboden durch die Zugabe von 10% Rinderserum und im flüssigem Nährboden durch die Zugabe von 0,25% Lecithin, Lanolin und Sonnenblumenöl inwieweit beeinflusst wird.

Das im Laufe dieser Untersuchungen verfolgte Verfahren wurde schon im Teil I unserer Mitteilungsserie[1] beschrieben, so können wir hier davon absehen.

Nicht nur die Nitro-phenole, sondern auch andere Nitro-Verbindungen mit den Struktur-Eigenschaften (1), (2), (3), (4) und (6) haben wir mit Rücksicht darauf untersucht, inwiefern sie in ihrer fungistatischen Wirkung durch Rinderserum beeinflusst werden können; die letzteren wurden allerdings nur auf Maische-Agar-Nährboden geprüft. Es scheint nämlich nicht ausgeschlossen zu sein, dass einige unter diesen Verbindungen bei biologischen Umständen nicht nur mit Sulfhydryl-Gruppen, sondern ausserdem auch mit anderen Atom-Gruppen (z.B. mit $-NH_2$, $-NH$-Radikalen, welche im Serum in bedeutender Menge anwesend sind) reagieren können; so mögen auch diese Reaktionen in ihrem fungistatischen Wirkungsmechanismus mitspielen.

Es ist aus der chemischen Fachliteratur bekannt, dass das 2 : 4-Dinitro-phenol (F/348) und das 2 : 4-Dinitro-6-methyl-phenol (F/351) die oxidative Phosphorilation spezifisch hemmen können.[25-27]



Insofern die Hemmung der oxidativen Phosphorilation im fungistatischen Wirkungsmechanismus des 2 : 4-Dinitro-phenols und des 2 : 4-Dinitro-6-methyl- phenols eine Rolle spielt, so muss ihre fungistatische Wirkung durch die Zugabe von Adenosintriphosphat zum Nährboden wesentlich vermindert oder gänzlich abgewehrt werden.

Zur Entscheidung dessen, ob die Hemmung der oxidativen Phosphorilation im fungistatischen Wirkungsmechanismus dieser Nitro-Verbindungen wirklich mitspielt, haben wir deren fungistatische Wirkung auf das *Penicillium simplicissimum* und das *Saccharomyces cerevisiae* auf festem Maische-Agar-Nährboden auch mit 0,1% Adenosin-triphosphat (Tetra-natrium Salz)-Gehalt einer Prüfung unterzogen; diese Untersuchungen haben wir auch auf andere Nitro-phenole (und zugleich auf einige andere Nitro-Verbindungen von verwandter Struktur) ausgebreitet.

Die bei der Untersuchung des Wirkungsmechanismus der Nitro-Verbindungen erhaltenen Resultate stehen in den Tabellen 4 und 5 zusammengefasst.

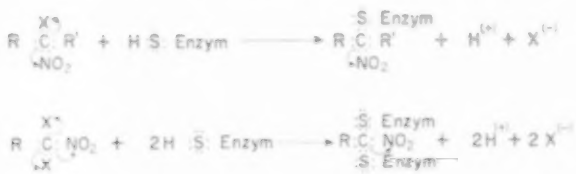
BESPRECHUNG DER ERGEBNISSE

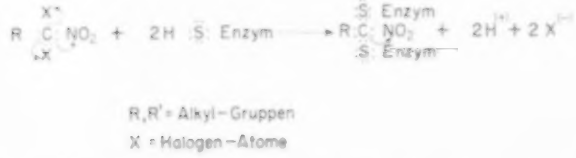
Durchschauend die Resultate unserer Untersuchungen kann man folgendes feststellen.

(1) Unter den aliphatischen und araliphatischen Nitro-Verbindungen sind nur jene fungistatisch wirksam, welche an demselben Kohlenstoffatom, wo sich ihre Nitro-Gruppe befindet, auch ein oder mehrere Halogen-Atome enthalten, oder aber diejenigen, die ihre Nitro-Gruppe an ein Kohlenstoffatom mit doppelter Bindung gebunden enthalten.

Jene aliphatischen und araliphatischen Nitro-Verbindungen, welche keine solche Struktur-Eigenschaften aufweisen können, bleiben wirkungslos.

Die Verbindungen mit Halogen-Atom-Gehalt unter den aliphatischen und araliphatischen Nitro-Verbindungen von der erwähnten Struktur sind fungistatisch deshalb wirksam, da die elektrophyle Eigenschaft der in ihren Molekülen anwesenden Nitro-Gruppe und des Halogen-Atoms (oder Atome) sich summiert, demzufolge wird jenes Kohlenstoffatom, woran die Nitro-Gruppe und das Halogen-Atom (Atome) gebunden wird, polarisiert; es wird nun dadurch fähig, auf Grund von kryptoionischen Mechanismus mit den Sulfhydryl-Gruppen von Elektron-Donor-Eigenschaft der Sulfhydryl-Enzyme der Pilz-Zellen in Reaktion zu treten, wobei Halogen- und Wasserstoff-Ione sich abtrennen. In anschaulicher Form:





R,R' = Alkyl–Gruppen

X = Halogen–Atome

(2) Jene Nitro-Verbindungen, bei denen die Nitro-Gruppe an ein Kohlenstoffatom von Doppelbindung angeschlossen ist, mögen ihre fungistatische Wirksamkeit wahrscheinlich ebenso durch ihre Reaktion mit den Sulfhydryl-Gruppen einzelner Sulfhydryl-Enzyme der Pilz-Zellen ausüben.

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TABELLE 4. DIE DURCH VERSCHIEDENE STOFFE VERURSACHTE BEEINFLUSSUNG DER AUF FESTEM MAISCHE-AGAR-NÄHRBODEN AUSGEÜBTEN FUNGISTATISCHEN WIRKUNG VON NITRO-VERBINDUNGEN

Nr.	Verbindungen	*Penicillium simplicissimum*					*Saccharomyces cerevisiae*				
		(ohne Zugaben)	10% Rinderserum	0·3% Cisteinhydrochlorid	0·3% Na-Thioglycolat	0·1% Adenosintriphosphat (Tetra-Na-Salz)	(ohne Zugaben)	10% Rinderserum	0·3% Cisteinhydrochlorid	0·3% Na-Thioglycolat	0·1% Adenosintriphosphat (Tetra-Na-Salz)
F/267	1-Nitro-1 : 1-dibrom-aethan	>90	>90	—	—		>90	>90	—	—	
F/268	1-Nitro-1 : 1-dibrom-propan	>90	>90	—	—		>90	>90	—	—	
F/277	1-Phenyl-2-nitro-aethylen	22	20	—	—		63	59	—	—	
F/281	1-(2'-Furyl)-2-nitro-aethylen	25	21	—	—		43	43	—	—	
F/282	1-(2'-Furyl)-2-methyl-2-nitro-aethylen	35	35	—	—		12	10	—	—	
F/284	1-Phenyl-2-nitro-1 : 2-dibrom-aethan	27	27	—	—		57	56	—	—	
F/290	1 : 3-Dinitro-benzol	55	53	55	54	56	12	12	10	13	12
F/291	2 : 4-Dinitro-toluol	20	17	20	19	20	10	10	10	10	10
F/292	3 : 5-Dinitro-toluol	20	20	19	19	21	15	14	14	15	15
F/294	2 : 4 : 6-Trinitro-toluol	27	10	20	18	25	20	17	15	15	19
F/315	2 : 4-Dinitro-chlor-benzol	40	37	20	22	39	30	25	17	15	31
F/316	2-Nitro-benzyl-chlorid	15	14	10	10		17	15	10	10	
F/317	2-Nitro-benzyl-jodid	30	28	11	12		37	32	12	12	
F/325	3-Nitro-phenol	28	15	16	—	25	—	—	—	—	—
F/326	4-Nitro-phenol	28	16	16	—	26	—	—	—	—	—
F/328	2-Nitro-4 : 6-dimethyl-phenol	20	13	15	—	21	12	—	—	—	14
F/334	2-Nitro-4-propionyl-phenol	20	15	17	12	18	18	10	12	—	20
F/335	2-Nitro-4-chlor-phenol	40	17	27	18	38	10	—	—	—	11
F/336	2-Nitro-4 : 6-dibrom-phenol	24	12	19	12	22	17	12	13	10	18
F/337	3-Nitro-4 : 6-dibrom-phenol	30	12	21	13	28	32	14	20	16	30
F/344	3-Nitro-4-hydroxy-benzoesäure-*n*-propyl-ester	20	13	20	15	22	10	10	10	10	10
F/348	2 : 4-Dinitro-phenol	17	10	16	11	16	10	10	10	10	10
F/349	2 : 5-Dinitro-phenol	45	22	18	—	42	25	15	18	—	25
F/351	2 : 4-Dinitro-6-methyl-phenol	20	16	15	—	21	22	16	20	—	20
F/352	2 : 4-Dinitro-5-methyl-phenol	26	15	15	—	25	17	10	10	10	18
F/356	2 : 4-Dinitro-6-chlor-phenol	40	22	19	10	38	18	12	10	—	18
F/402	2-Nitro-4-methyl-anilin	35	33	32	34		10	10	10	—	
F/403	2-Nitro-4-aethoxy-anilin	35	35	32	33		—	—	—	—	
F/404	2-Nitro-4-chlor-anilin	18	16	18	16		—	—	—	—	
F/422	2 : 4-Dinitro-anilin	40	38	41	40	41	30	30	30	29	28

Siehe Zeicherklärung der Tabelle 3.

TABELLE 5. DIE BEEINFLUSSUNG DER AUF DAS *Penicillium simplicissimum* AUSGEÜBTEN FUNGISTATISCHEN WIRKUNG DER NITRO-PHENOLE DURCH DIE ZUGABE VON VERSCHIEDENEN SUBSTANZEN ZUM FLÜSSIGEN MAISCHE—NÄHRBODEN

Nr.	Verbindungen	———	10% Rinder-serum	0·25% Lecithin	0·25% Lanolin	0·25% Sonnen-blumenöl
F/324	2-Nitro-phenol	10 000	5000	10 000	10 000	10 000
F/325	3-Nitro-phenol	5000	5000	5000	5000	5000
F/326	4-Nitro-phenol	5000	5000	5000	5000	5000
F/334	2-Nitro-4-propionyl-phenol	10 000	(5000)	10 000	10 000	10 000
F/335	2-Nitro-4-chlor-phenol	50 000	10 000	50 000	50 000	25 000
F/336	2-Nitro-4 : 6-dibrom-phenol	10 000	10 000	10 000	10 000	10 000
F/337	3-Nitro-4 : 6-dibrom-phenol	10 000	10 000	10 000	10 000	10 000
F/348	2 : 4-Dinitro-phenol	5000	(5000)	5000	5000	5000
F/349	2 : 5-Dinitro-phenol	50 000	10 000	50 000	25 000	50 000
F/351	2 : 4-Dinitro-6-methyl-phenol	10 000	10 000	10 000	5000	10 000
F/352	2 :4-Dinitro-5-methyl-phenol	5000	5000	5000	5000	5000
F/353	2 : 6-Dinitro-4-methyl-phenol	5000	(5000)	5000	5000	5000
F/356	2 : 4-Dinitro-6-chlor-phenol	10 000	5000	10 000	10 000	10 000
F/357	2 : 6-Dinitro-4-chlor-phenol	5000	5000	5000	5000	5000

Siehe Zeichenerklärung der Tabelle 1.

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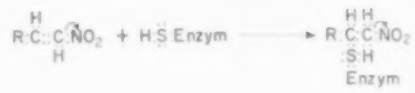
Die Doppelbildung von Elektron-Donor-Eigenschaft dieser Verbindungen wird durch die elektrophyle Nitro-Gruppe recht stark polarisiert; infolgedessen werden diese Verbindungen geeignet, mit den SH-Gruppen der Sulfhydryl-Enzyme nach folgendem Schema in Reaktion zu treten:



Die Intensität der *in vitro* ausgeübten fungistatischen Wirksamkeit der Verbindungen dieses Typus wird nach aller Wahrscheinlichkeit durch den Grad ihrer Affinität gegen die Sulfhydryl-Gruppen bestimmt.

Der Grad ihrer Affinität zu den Sulfhydryl-Gruppen irgendwelches lebenswichtigen Sulfhydryl-Enzyms der Pilz-Zellen ist von der Natur des in ihren Molekülen anwesenden R-Radikals abhängig, ferner von dem Umstand, ob an die ungesättigten Kohlenstoffatome H-Atome oder irgendwelche anderen Atom-Gruppen sich anschliessen.

(3) Die Richtigkeit unserer Voraussetzung über den fungistatischen Wirkungsmechanismus der Halogen-Atom (Atome) sowie ungesättigte Bindung enhaltenden aliphatischen und araliphatischen Nitro-Verbindungen scheint unsere Erfahrung zu rechtfertigen, dass einige stark fungistatisch wirkende Prototypen derselben (F/267, 268, 277, 281, 282, 284) auf einem Cystein-hydrochlorid oder Na-Thioglycolat enthaltenden festen Maische-Agar Nährboden ganz wirkungslos bleiben; sie erweisen sich aber fungistatisch bedeutend wirksam auf einem festen Nährboden der von diesen Sulfhydryl-Verbindungen frei ist.

Daraus kann man den Schluss ziehen, dass das Cystein-hydrochlorid, bzw. das Na-Thioglycolat im Nährboden mit ihnen extracellulär in Reaktion tritt und diese in für weitere ähnliche Reaktion unfähige Verbindungen unwandelt; dadurch werden die Pilz-Zellen gegen deren Einwirkung gleichwie in Schutz genommen.

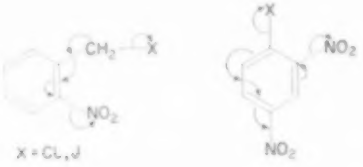
Die infolge der Einwirkung dieser Verbindungen *schon erfolgte* Hemmung der Vermehrung des *Penicillium simplicissimum* und *Saccharomyces cerevisiae* kann von vornherein weder das Cystein-hydrochlorid noch das Na-Thioglycolat aufheben, da die intercellulare Reaktion zwischen den Halogen-Atome oder ungesättigte Bindung enthaltenden aliphatischen und araliphatischen Nitro-Verbindungen sowie den SH-Gruppen der lebenswichtigen Sulfhydryl-Enzyme der Pilz-Zellen irreversibel ist. Da die Reversibilität der schon stattgefundenen intercellularen Reaktion durch das Cystein-hydrochlorid oder das Na-Thioglycolat *unter physiologischen Umständen für grundsätzlich ausgeschlossen gelten kann*, haben wir derartige Versuche auch nicht unternommen.

(4) Die Ergebnisse unserer Wirkungsmechanismus-Untersuchungen beweisen, dass die auf festem Nährboden ausgeübte fungistatische Wirkung des 2-Nitro-benzylchlorids und -jodids auf das *Penicillium simplicissimum* und *Saccharomyces cerevisiae* sowohl das Cysterin-hydrochlorid als auch das Na-Thioglycolat weitgehend abwehren kann, die Wirkung des 2 : 4-Dinitro-chlor-benzols aber bedeutend vermindert.

Daraus folgt, dass das 2-Nitro-benzyl-chlorid, das 2-Nitro-benzyl-jodid und das 2 : 4-Dinitro-chlor-benzol ihre fungistatische Wirkung gleichfalls als "Sulfhydryl-Enzym-Inhibitors" ausüben.

Diese Ansicht wird prinzipiell durch die Tatsache begründet, dass die Nitro-Gruppen durch ihre starke elektrophyle Eigenschaft die Elektronen-Dichte um jenes Kohlenstoffatom des Moleküls vermindern, an welches Atom das gleichfalls elektrophyle Halogen-Atom gebunden ist; auf diese Weise wird es stark polarisiert und zur Teilnahme an kyptoionischen Reaktionen geeignet gemacht.

In anschaulicher Form lässt sich der Vorgang so darstellen:



(5) Das Cystein-hydrochlorid oder das Na-Thioglycolat beeinflussen die fungistatische Wirkung des 1 : 3-Dinitro-benzols, des 2 : 4- und 3 : 5-Dinitro-toluols nicht (sie vermindern diese Wirkung jedoch bei dem 2 : 4 : 6-Trinitro-toluol nur einigermassen); deshalb ist es wahrscheinlich zu halten, dass diese nicht durch die gelegentliche Lähmung der Funktion von Sulfhydryl-Enzymen in den Stoffwechsel der Pilz-Zellen eingreifen. Sowohl diese, als auch die anderen untersuchten fungistatisch wirkenden Nitro-Derivate des Benzols, Naphtalins, Diphenyls und Phenanthrens sollen ihre fungistatische Wirksamkeit vermutlich auf Grund eines ganz anderen Mechanismus ausüben.

Es ist allgemein bekannt, dass zehlreiche Nitro- und Polynitro-Verbindungen mit recht mannigfaltigen organischen Verbindungen Molekül-Assoziaten, Molekül-Verbindungen von verschiedener Struktur zu bilden vermögen.[28–32]

Wir müssen dabei annehmen, dass unter den bei unseren Versuchen fungistatisch erwiesenen aromatischen Nitro-Verbindungen diejenigen, die ausser der Nitro-Gruppe in ihren Molekülen keine anderen Reaktiven Gruppen enthalten, ihre fungistatische Wirkung dadurch entfalten, dass die mit lebenswichtigen Eiweissen, Lipoiden, Lipoproteiden, Nukleoproteiden der Pilz-Zellen, bzw. mit ihren gewissen Kettenteilen und Gruppen Molekül-Verbindungen, Assoziaten bilden, diese dadurch denaturieren und zur Ausübung ihrer normalen biochemischen Funktion unfähig machen. Zur Bestetigung dieser Voraussetzung werden wir im späteren eingehendere Untersuchungen vornehmen.

Die Nitro-Derivate einiger mehrkerniger aromatischer Kohlenwasserstoffe (z.B. das F/296, 301, 302, 303, 304, 305, 320, 321, 322, 323) können trotzdem, dass sie—wenigstens unter Laboratoriumsumständen—mit verschiedenen Verbindungen Molekül-Assoziaten zu bilden vermögen, doch keine fungistatische Wirkung ausüben. Die Ursache ihrer Wirkungslosigkeit ist unseres Erachtens darin zu suchen, dass sie infolge ihrer geringen Lösbarkeit im Wasser, sowie ihrer bedeutenden Disposition zur Aggregation nicht einmal in Kolloid-Zustand jenes Mindestmass der Konzentration im Nährboden erreichen können, das zur Herbeiführung der Fungistase notwendig ist.

(6) Die Nitro-Derivate der Benzoesäure, Phenyl-Essigsäure und der 1-Naphtyl-Essigsäure (F/394, 395, 396, 397, 398), und auch die Pikrinsäure, bleiben vermutlicherweise deshalb wirkungslos, weil sie wegen ihres starken saurigen Charakters die Wand der Pilz-Zellen nicht durchdiffundieren und in deren Zwischenzellenraum nicht eindringen können.

(7) Serum kann auf festem Maische-Agar-Nährboden nur die fungistatische Wirkung der Nitro-phenole und des 2 : 4 : 6-Trinitro-toluols erheblich beeinflussen.

Auch das Cystein-hydrochlorid und das Na-Thioglycolat vermindern im allgemeinen in bedeutendem Masse die fungistatische Wirkung der Nitro-phenole. Diese Wirkungsabnahme infolge des Cystein-hydrochlorids und Na-Thioglycolats kann man aber nicht als Folge einer zwischen ihnen und den Nitro-phenolen zustande gekommenen spezifischen Reaktion betrachten, sondern sie ist wohl der Reduktion ihrer Nitro-Gruppe zuzuschrieben, die durch das Cystein-hydrochlorid, bzw. das Na-Thioglycolat verursacht wurde.

Im Wirkungsmechanismus der untersuchten und fungistatisch erwiesenen Nitrophenole mag auch die Hemmung der Funktion der Sulfhydryl-Enzyme der Pilz-Zellen infolge ihrer aspezifisch oxidierenden Wirkung einen Anteil haben; es ist aber wahrscheinlich, dass es nicht die Hauptkomponente ihres Wirkungsmechanismus bildet.

Auf flüssigem Nährboden kann das Rinderserum nur die fungistatische Wirkung des 2-Nitro-4-chlor-phenols und des 2 : 5-Dinitro-phenols wesentlich vermindern, die des 2-Nitro-phenols, des 2-Nitro-4-propionyl-phenols und des 2 : 4-Dinitro-6-chlor-phenols nur in geringem Masse, die der übrigen Nitro-phenole aber durchaus nicht. Lecithin, Lanolin und Sonnenblumenöl können die fungistatische Wirkung deren Mehrheit überhaupt nicht, die der Minderheit auch nur wenig beeinflussen.

Die fungistatische Wirkung der Nitro-phenole auf festem Nährboden wird durch die Zugabe von Adenosin-triphosphat zum Nährboden nur unbedeutend vermindert (Ebensonwenig die der Dinitro-benzol-Derivate und des 2 : 4-Dinitro-anilins). Daraus ergibt sich die Folge, dass diese Verbindungen ihre fungistatische Wirkung nicht durch die Hemmung der oxidativen Phosphorilation ausüben können.

Wenn wir all dies berücksichtigen, können wir zum Schluss kommen, dass der fungistatische Wirkungsmechanismus der Nitro-phenole viel komplizierter ist, als derjenige der einfachen Phenol-Derivate oder Halogen-phenole.[1] In ihrem Wirkungsmechanismus müssen verschiedene Faktoren mitwirken: die Denaturierung der Eiweisse der Pilz-Zellen, die oxidative Inaktivierung einzelner ihrer Sulfhydryl-Enzyme, ausserdem noch vermutlich auch die Bildung einer Molekül-Assoziation mit einzelnen Zellbestandteilen.

Die Frage, welcher biochemische Reaktionstypus in der Herbeiführung der Fungistase durch irgendein Nitro-phenol am entscheidendsten mitwirkt, hängt weitgehend von der speziellen chemischen Struktur des betreffenden Nitro-phenols ab, sowie von dessen Dissoziationskonstante, von der Elektronen—Dichte um einzelne Zentren des Moleküls, von der Qualität der polaren Gruppen des Moleküls und von der Stellung dieser Gruppen im Verhältnis zueinander und offenbar auch von anderen, bisher noch nicht bekannten Faktoren.

(8) Unter den Nitro-anilin-Derivaten sind nur die 4-Methyl-, 4-Äthoxy-, 4-Chlor-, und 4-Nitro-Derivate des 2-Nitro-anilins fungistatisch wirksam. Es ist interessant, dass das 2-Nitro-anilin selbst keine bedeutende Wirkung ausüben kann, sondern nur

dessen 4-substituierte Derivate. Ihre fungistatische Wirkung wird durch Serum, Cystein-hydrochlorid und Na-Thioglycolat nicht beeinflusst. Ihr Wirkungsmechanismus ist uns nicht bekannt.

Zusammenfassung—Vertasser hat die fungistatische Wirkung von 184 Nitro-Verbindungen auf einem 10% Rinderserum enthaltenden flüssigen und 3% Agar enthaltenden festen Maische-Agar-Nährboden untersucht und unter diesen auch den Wirkungsmechanismus der am wirksamsten erwiesenen studiert. Er suchte in der Gruppe der Nitro-Verbindungen Zusammenhänge zu finden einerseits zwischen der chemischen Struktur und der fungistatischen Wirksamkeit, andererseits aber zwischen der chemischen Struktur und dem Wirkungsmechanismus.

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ACTION DU 2-PAM ET DES INHIBITEURS DE L'ACETYLCHOLINESTERASE SUR LE CHOC ANAPHALACTIQUE DU LAPIN

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(*Received* 7 *September* 1960)

Abstract—DFP and eserine have certain antiallergic properties. These are not a result of blocking of the neuromuscular junctions, but of deactivation by an enzyme of an esterase character. Our results do not allow us to decide whether this enzyme is an integral part of the "complement", or a distinct molecular structure.

2-PAM (a very specific reactivator of acetylcholinesterase that has been inhibited by alcoyl-phosphates) is able to suppress the protective action of DFP. This suggests that the configuration of the active centres of the enzyme are very close to that of acetylcholinesterase. We propose to name this enzyme *anaphylesterase*.

LORSQUE dans le plasma ou le sérum d'un animal sensibilisé, les antigènes se combinent aux anticorps correspondants, le complément se fixe aux complexes ainsi formés.

Le complément est également fixé à la surface des cellules porteuses de pareils complexes. Il en résulte une modification de la perméabilité de la membrane, qui se traduit, notamment, par une diminution de la concentration intracellulaire en K et en acides aminés, et s'il s'agit des hématies, par une perte d'hémoglobine (hémolyse).[1] Ces différents effets sont donc le reflet de l'activité du complément.

Comme l'ont montré Levine[2] et Becker[3], les inhibiteurs des estérases, en particulier les alcoyl-phosphates, atténuent *in vitro* le décours de certaines réactions immunitaires, telle l'augmentation de perméabilité, responsable de l'hémolyse des hématies sensibilisées. C'est pourquoi ces auteurs concluent que certains composants du complément sont doués de caractères propres aux estérases. D'ailleurs, les cellules sensibilisées qui ont fixé le complément ont acquis la propriété d'hydrolyser le *p*-toluènesulfonylargininemethyl ester (TAMe).[3] En outre, certains constituants du complément, une fois purifiés, hydrolysent l'acétyltyrosyléthyl ester.[4, 5]

Puisque l'une des activités enzymatiques d'origine complementaire semble être celle d'une estérase, nous avons recherché si, d'une part, les alcoyl-phosphates sont capables *in vivo* de modifier le décours du choc anaphylactique et si, d'autre part, l'iodure de N-méthylpyridine-2-aldoxime (2-PAM), réactivateur spécifique de l'acétylcholinestérase inhibée par les alcoyl-phosphates,[6] est susceptible de perturber les effects eventuels du dérivé organophosphoré. Wilson[7] a, en effet, montré que les

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fonctions possédant un caractère nucléophilique (fonction amine, pyridyl, hydroxyl, sulfhydryl, etc.) réactivent l'acétylcholinestérase phosphorylée.

METHODES

Les lapins ont été sensibilisés 3 semaines avant l'expérience par administration sous-cutanée de 5 ml de blanc d'oeuf de poule, pendant 3 jours consécutifs.

Le jour de l'expérience, les lapins sont anesthésiés au Nembutal, par voie intraveineuse, à raison de 30 mg/kg. Après trachéotomie, la respiration artificielle est instaurée d'emblée. La pression artérielle est enregistrée à la carotide.

Les lapins, d'abord injectés par voie intraveineuse, de sulfate d'atropine à raison de 3 mg/kg, ont été ensuite répartis en trois groupes. Le premier est traité par le di*iso*-propylfluoro phosphate de soude (DFP) injecté par voie intraveineuse à doses fractionnées jusqu' à un total de 4 mg/kg.

Le second groupe est également traité par le DFP. Lorsque les symptômes de l'intoxication sont installés depuis quelques minutes, l'animal reçoit en injection intraveineuse de l'iodure de N-méthylpyridine-2-aldoxime (2-PAM, pyridine-2-aldoxime methiodide) à raison de 75 mg/kg. Cette substance définie par Wilson, débloque de manière instantanée l'acétylcholinestérase inactivée par les alcoylphosphates.[8–11]

Le troisième groupe de lapins sensibilisés est injecté de physostigmine (ésérine) à doses fractionnées, jusqu'à un total de 4 mg/kg. Dans une expérience contrôle, un lapin de ce groupe a été traité par 2-PAM aprés l'installation de l'intoxication ésérinée.

Quinze à vingt minutes après la dernière injection, soit de l'inhibiteur, soit du 2-PAM, l'antigène est administré sous la forme d'une solution de blanc d'oeuf dilué au quart par NaCl 0,9%, à raison de 1 ml/kg.

Les variations de la pression artérielle générale permettent de juger de l'intensité des réactions anaphylactiques. Celles-ci sont d'autant plus importantes que l'hypotension est plus profonde et plus durable.

RESULTATS

(1) L'administration de DFP, comme celle de physostigmine, entraîne par blocage de l'acétylcholinestérase et accumulation subséquente d'acétylcholine l'apparition de symptômes nombreux et divers. Les uns sont de nature muscarinique, tels la salivation, la bradycardie et le miosis; ils sont inhibés par l'atropine. Les autres, nicotiniques, parmi lesquels la fibrillation musculaire généralisée, ne sont pas supprimés par cet alcaloïde. Cette fibrillation disparaît lorsque l'accumulation progressive d'acétylcholine a finalement bloqué les récepteurs de synapses neuromusculaires. La disparition de toute coordination neuromusculaire par ce blocage synaptique entraîne l'arrêt de la respiration.

Puisque les lapins sensibilisés dont nous étudions les réactions sont d'emblée soumis à la respiration artificielle et traités par l'atropine, ils sont donc protégés des effets immédiats, muscariniques et nicotiniques, de l'intoxication par le DFP ou par l'ésérine. L'acétylcholinestérase est évidemment inactivée.

(2) Les lapins sensibilisés intoxiqués par le DFP ou la physostigmine prèsentent, lors de l'administration intraveineuse de l'antigène, une chute tensionnelle peu importante, de durée brève. Les propriétés anti-anaphylactiques de l'ésérine sont plus

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marquées que celle du DFP (Tableau 1). Les inhibiteurs des estérases se montrent ainsi doués d'une certaine activité anti-allergique. Celle-ci, toutefois, n'est pas suffisante pour supprimer entièrement toute manifestation tensionnelle d'origine anaphylactique, comme le montre la Fig. 1.

(3) L'administration de 2-PAM au lapin préalablement traité par l'ésérine ne modifie pas l'intoxication que provoque alors le blocage de l'acétylcholinestérase. La chute de

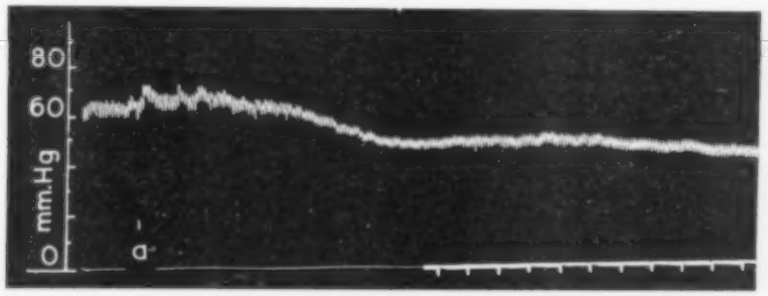

FIG. 1. Effet anti-anaphylactique de l'ésérine.

Enregistrement de la pression artérielle carotidienne d'un lapin de 2 kg 100, anesthésié au nembutal par voie intraveineuse et soumis à la respiration artificielle. Temps en minutes.

L'animal a été sensibilisé par l'administration de blanc d'oeuf, 3 semaines auparavant. Dès la fin de l'anesthésie, il a reçu 3 mg/kg d'atropine, puis de l'ésérine à raison de 4 mg/kg.

En *a* s'effectue l'administration déchainante de blanc d'oeuf. Elle n'est suivie d'aucune chute tensionelle immédiate: l'animal est protégé des effets de la réaction anaphylactique.

TABLEAU 1. ACTION DES INHIBITEURS DES ESTÉRASES SUR L'HYPOTENSION ANAPHYLACTIQUE

Conditions	Pression artérielle (% de la valeur -contrôle)	Durée de l'hypotension minutes	Nombre d'observations
+ DFP	40	12	5
+ DFP + PAM	10	25	4
+ ésérine	50	10	4

Les lapins sont sous respiration artificielle et reçoivent de l'atropine (3 mg/kg). Le DFP, l'ésérine et le 2-PAM sont injectés, en doses fractionnées, par voie intraveineuse. La pression artérielle est enregistrée à la carotide. Les chiffres du tableau expriment en % du controle, la valeur la plus basse obtenue lors de l'injection intraveineuse de l'antigène.

pression artérielle qui suit l'injection de l'antigène reste alors peu importante et de très courte durée. Ce résultat s'explique facilement: le 2-PAM ne réactive l'acétylcholinestérase que lorsque cet enzyme a été inhibé par un organophosphoré. Ce résultat est toutefois important, puisqu'il démontre que c'est bien au niveau d'une activité estérasique que le DFP et l'ésérine exercent leur action anti-anaphylactique.

(4) L'administration de 2-PAM aux lapins intoxiqués par le DFP entraîne immédiatement la disparition des fasciculations musculaires et restaure la force de contraction des muscles respiratoires. L'hydrolyse de l'acétylcholine est de nouveau possible. Le

blocage synaptique est aboli et la respiration artificielle peut alors être suspendue sans danger.

(5) Les lapins sensibilisés, d'abord traités par le DFP, ensuite par le 2-PAM, se trouvent, en ce qui concerne l'acétylcholinestérase, dans un état analgogue à celui des animaux qui n'auraient reçu que de l'atropine. Quand de l'antigène préparant leur est injecté par voie intraveineuse, ils présentent, après une latence de 30 à 45 sec, une chute brutale (7 cm Hg) et durable (25 min) de la pression artérielle, caractéristique du choc anaphylactique (Fig. 2). L'examen nécropsique du coeur révèle d'ailleurs la turgescence pathognomonique de l'artère pulmonaire.

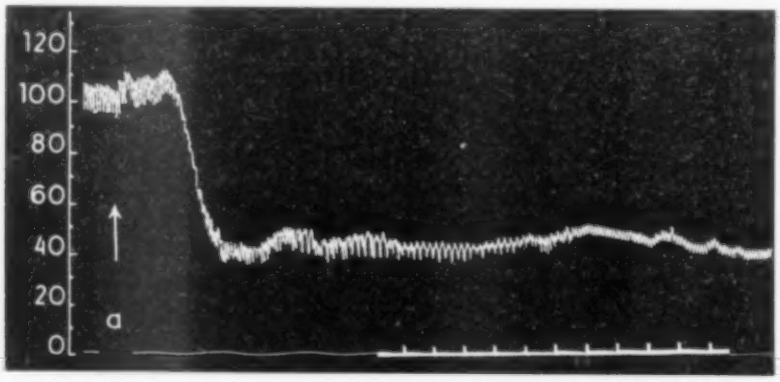

FIG. 2. Absence d'effet anti-anaphylactique du traitement DFP/2-PAM.

Enregistrement de la pression artérielle carotidienne chez un lapin sensibilisé (2 kg), anesthésié et soumis à la respiration artificielle (conditions identiques au lapin qui a servi à obtenir la Fig. 1).

Après injection d'atropine, il a reçu du DFP (4 mg/kg) jusqu'à apparition des signes d'intoxication, notamment fasciculation musculaire. A ce moment, injection intraveineuse rapide de 2-PAM (75 mg/kg) entrainant la disparition des signes toxiques.

En *a* s'effectue l'administration déchainante du blanc d'oeuf. Elle provoque alors une chute tensionelle immédiate suivie d'un collapsus durable. L'animal n'est pas protégé.

Ce résultat est à rapprocher de l'observation de Becker[12] montrant que l'acide hydroxamique restaure, *in vitro*, l'activité du complément inhibée par le DFP, puisque, comme l'a démontré Wilson[7], c'est par son caractère nucléophilique que l'acide hydroxamique réactive l'acétylcholinestérase phosphorylée.

Quelques expériences supplémentaires effectuées à titre de contrôle nous ont montré que chez les lapins sensibilisés dont la pression artérielle a été au préalable fortement abaissée par l'administration intraveineuse de l'antigène préparant, l'injection intraveineuse d'atropine (4 mg/kg) ne provoque pas la récupération des valeurs tensionnelles normales.

L'atropine, à elle seule, s'avère ainsi incapable de modifier le décours du choc de sensibilisation.

DISCUSSION

(1) L'atropine administrée à doses élevées ne prévient ni ne corrige l'hypotension artérielle générale d'origine anaphylactique. Puisque cet alcaloïde entre en compétition avec l'acétylcholine ou les autres substances cholinergiques, pour les récepteurs

des fibres musculaires lisses, on peut conclure qu'au cours de la réaction antigène–anticorps, il n'y a pas de libération de corps à activité cholinergique. La nature du mécanisme responsable de l'activation anaphylactique des fibres musculaires lisses doit donc être cherchée ailleurs que dans la libération d'une substance acétylcholinomimétique. D'ailleurs, les manifestations cardiaques d'origine allergique qui accompagnent la chute de la pression artérielle ne sont pas comparables à celles qui témoignent de l'accumulation périphérique d'un corps cholinergique.[13]

(2) Bien qu'ils s'avèrent incapables de supprimer complètement la chute tensionnelle d'origine anaphylactique, le DFP et l'ésérine sont doués d'une certaine activité anti-allergique. Celle-ci, qualitativement, est beaucoup moins importante que celle des diphénylpyrazolones. Elle dépend vraisemblablement de la mise hors d'action de certains processus secondaires participant accessoirement au déclenchement du choc anaphylactique.

Les effets anti-anaphylactiques du DFP et de l'ésérine ne peuvent pas dépendre d'une action au niveau des récepteurs des jonctions neuromusculaires, puisque ces derniers ne paraissent pas être intéressés directement dans la pathogénie du choc. Ces effets ne peuvent pas davantage s'expliquer par l'inhibition de l'acétylcholinestérase au niveau des synapses.

Il est donc nécessaire de postuler que ces inhibiteurs inactivent un autre enzyme à fonction estérasique. Comme cet enzyme, lorsqu'il est inhibé par un alcoyl-phosphate, est réactivé par un antidote aussi spécifique de l'acétylcholinestérase que le 2-PAM, nous devons conclure que la configuration des centres actifs de cet enzyme doit être très voisine de celle de l'acétylcholinestérase. Cet enzyme est-il le complément ou s'agit-il d'une entité chimique distincte? Nos résultats ne permettent pas de tirer de conclusions à ce sujet. Mais, ce qui paraît établi, c'est que l'activité d'un enzyme à fonction estérasique semble impliquée à titre accessoire dans la pathogénie du choc.

(3) Nous proposons d'appeler cet enzyme "anaphylestérase". Cette nouvelle appellation ne fait pas double emploi avec celle de "complément", même si des travaux ultérieurs démontraient qu' "anaphylestérase" et "complément" désignent une même structure. Le terme d' "anaphylestérase" nous parait, en effet ,plus conforme que le terme de "complément" aux règles de la nomenclature enzymologique. De plus, il a l'avantage de définir plus précisément un des mécanismes biochimiques responsable du choc anaphylactique, à savoir: l'hydrolyse d'une liaison ester.

RESUME

Le DFP et l'ésérine sont doués de certaines propriétés anti-allergiques. Cette action n'est pas le résultat d'un blocage des jonctions neuromusculaires, mais dépend de l'inactivation d'un enzyme à caractère estérasique. Cet enzyme fait-il partie intégrante de ce qu'on appelle généralement le complément, ou s'agit-il d'une structure moléculaire distincte? Nos résultats ne permettent pas de tirer de conclusions à ce sujet. Le fait que le 2-PAM, un réactivateur très spécifique de l'acétylcholinestérase inhibée par les alcoyl-phosphates, est capable de supprimer l'action protectrice du DFP, suggère que la configuration des centres actifs de l'enzyme est très voisine de celle de l'acétycholinestérase. C'est pourquoi nous proposons d'appeler cet enzyme l' "anaphylestérase", dénomination conforme aux règles de la nomenclature enzymologique. Cette nouvelle appellation a également l'avantage de définir plus précisément un des

mécanismes biochimiques responsable du choc anaphylactique, à savoir: l'hydrolyse d'une liaison ester.

Remerciements—Nous remercions le Dr. D. Nachmansohn, professeur de Biochimie à la Columbia University, New-York, d'avoir bien voulu nous fournir le 2-PAM utilisé dans ces expériences.

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BIOCHEMICAL EFFECTS OF DITHIAZANINE ON THE CANINE WHIPWORM, *TRICHURIS VULPIS**

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(*Received* 25 *July* 1960)

Abstract—Lactic, acetic, propionic and *n*-valeric acids, carbon dioxide, and small quantities of formic and *n*-butyric acids, are products of the aerobic and anaerobic metabolism of adult *Trichuris vulpis*, the canine whipworm. Approximately 50 per cent of the glucose taken up by this parasite is accounted for by these products. The parasite is not dependent on aerobic metabolism. In an atmosphere containing CO_2 (from 2 to 5 per cent) in nitrogen, *T. vulpis* survives *in vitro* for considerably longer periods and metabolizes carbohydrate at a higher rate than in nitrogen alone, in air, or in a mixture of CO_2 (5 per cent) and oxygen (20 or 95 per cent).

Dithiazanine, a cyanine dye effective in the treatment of canine and human whipworm infections, in concentrations which do not affect the motility of *T. vulpis*, produces an irreversible inhibition of the uptake of glucose by the parasite and a marked reduction in the concentrations of free glucose and ATP, and in the size of the carbohydrate stores in the worm. It is concluded that dithiazanine interferes with the transport of glucose in *T. vulpis*. As a result, utilization of endogenous carbohydrate is increased. Depletion of the carbohydrate stores and inability to utilize exogenous glucose brings about a decrease in the generation of energy-rich phosphate bonds. These biochemical changes can account for the chemotherapeutic action of dithiazanine in trichuriasis.

RECENT reports have demonstrated the chemotherapeutic effectiveness of a cyanine dye, dithiazanine, in the treatment of human subjects infected with the whipworm, *Trichuris trichiura*,[1] as well as with other intestinal nematodes (*Strongyloides stercoralis*, *Enterobius vermicularis* and *Ascaris lumbricoides*).[2, 3] In the course of investigations designed to determine the biological properties of cyanine dyes[4] the anthelmintic activity of these compounds was uncovered during World War II.[5–7] In extremely low concentrations (5×10^{-8} M) cyanines inhibit the oxygen uptake of a number of helminths.[8–11] This effect accounts for the chemotherapeutic activity of cyanines against the filarial worm *Litomosoides carinii*. The structural property conferring these metabolic and chemotherapeutic actions of cyanines is the amidinium ion resonance system which is a common characteristic of this group of compounds.[12] Subsequent investigations have revealed that cyanine dyes have chemotherapeutic

* This investigation was conducted under the auspices of the Commission on Parasitic Diseases of the Armed Forces Epidemiological Board and was supported in part by the Office of the Surgeon General of the Army and by research grants (E-668 and E-2347) from the National Institutes of Health, U.S. Public Health Service.

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activity against certain intestinal nematodes of dogs (*Ascaris*, *Trichuris* and *Ancylostoma*),[13] of rats and of mice (pinworms, strongyloids).[14] These observations led to the development of a cyanine dye, pyrvinium chloride, which proved highly effective in the treatment of human pinworm infections.[15, 16] Since the oxygen tension of the habitat of these helminths is extremely low and since most of the above-mentioned parasites depend for survival on anaerobic, rather than on aerobic, metabolism[17, 18] it appears that the chemotherapeutic actions of cyanine dyes on these organisms are brought about by a mechanism other than inhibition of oxidative reactions. The high activity of dithiazanine against similar types of intestinal nematodes provides additional support for this conclusion. In the present study an attempt has been made to determine the effects of dithiazanine on the anaerobic metabolism of *Trichuris vulpis*, the canine whipworm highly susceptible to the chemotherapeutic actions of this cyanine dye.

The observations reported below indicate that under both anaerobic and aerobic conditions sublethal concentrations of dithiazanine irreversibly inhibit the transport of glucose into *Trichuris vulpis*. Lack of availability precluded similar studies using the human whipworm, *Trichuris trichiura*.

METHODS AND MATERIALS

After removal of the whipworms from the caecal mucosa of dogs, the parasites were placed in a medium which had the following composition: 0·12 M NaCl, 0·005 M KCl, 0·001 M $CaCl_2$, 0·001 M $MgCl_2$, 0·02 M Tris*buffer (pH 7·7), 0·002 M sodium phosphate buffer (pH 7·7), 0·05 M glucose; penicillin, streptomycin and kanamycin, each in a concentration of 10 μg per ml; and mycostatin, 1 μg per ml. Subsequently, this medium will be referred to as the basic medium. The nematodes were freed of contaminating intestinal contents and tissue debris by three transfers into petri dishes containing the basic medium, and were then blotted (using Whatman filter paper no. 50), weighed, and placed in solutions in which their metabolic activities were to be determined. One milliliter of medium was used per worm. When CO_2 was used in the atmosphere, sodium bicarbonate was added to the basic medium, in order to readjust the pH to 7·7. The final concentration of $NaHCO_3$ was $4{\cdot}5 \times 10^{-2}$ M with 5 per cent CO_2, $1{\cdot}3 \times 10^{-2}$ M with 2 per cent CO_2 and 6×10^{-4} M with 0·3 per cent CO_2. With 0·1 per cent CO_2 no bicarbonate was required, since the decrease in the pH produced by this concentration of gas was not significant. Gassing and incubation were carried out in the conventional Warburg apparatus at 37 °C. Glucose was determined by the method of Somogyi[19], lactic acid according to Barker and Summerson[20], glycogen according to Hassid and Abraham[21] and total carbohydrate according to a method used in a previous study.[8] Volatile acids were isolated from the media by distillation *in vacuo* and subsequent concentration, as described previously;[22] they were then separated and tentatively identified by gas partition chromatography, according to James and Martin[23].

Survival of *T. vulpis in vitro* was determined at 37 °C by incubating each worm in a screw-capped culture tube containing 5 ml of medium. Each experimental condition employed five worms and transfer to fresh media was made every three or four days.

* In this paper the following abbreviations are used: ATP, adenosine triphosphate; Tris, tris-hydroxymethylamino methane; TPN, triphosphopyridine nucleotide.

When gas mixtures other than air were used, the medium in each tube was gassed for 1 min before, and for another minute after the parasite had been introduced. This was followed immediately by sealing the screw-capped culture tube. The worms were observed daily for motility, which was used as the criterion for survival.

Concentrations of glucose and of ATP in the worms were determined enzymatically, as proposed by Slein *et al.*[24] by measuring the reduction of TPN as a result of the combined actions of hexokinase and of glucose-6-phosphate dehydrogenase. For these analyses the parasites were homogenized with cold perchloric acid (final concentration, 4 per cent). The mixtures were centrifuged at 1000 *g* for 15 min and measured volumes of the supernatant fluids were adjusted with KOH (2 N) to a pH of 7·0. The insoluble potassium perchlorate was removed by centrifugation at 1000 × *g* for 15 min. All operations were carried out at temperatures between 0 and 4 °C. Glucose was determined in aliquots of the neutralized supernatant fractions in a reaction mixture containing 0·01 M $MgCl_2$, 0·05 M potassium glycylglycine buffer (pH 7·4), 0·0003 M TPN and 0·003 M ATP (pH 7·4) in a final volume of 1 ml. After glucose-6-phosphate dehydrogenase was added, the change in optical density was determined at 340 mμ in a Beckman DU spectrophotometer. On completion of the reaction, hexokinase was added and the reduction of TPN was measured again in the same manner in order to determine free glucose. The same procedure was used for the determination of ATP, except that glucose (final concentration: 0·0015 M) was added to the reaction mixture in place of ATP. Under these conditions, reduction of 1·0 μmole of TPN is equivalent to 1·0 μmole of glucose if an excess of ATP is provided, and to 1 μmole of ATP in the presence of an excess of added glucose. Addition of glucose-6-phosphate dehydrogenase prior to that of hexokinase insures the dehydrogenation of any glucose-6-phosphate which may be present initially in the reaction mixture. Crystalline hexokinase was prepared according to an unpublished method of Darrow and Colowick[25]. Glucose-6-phosphate dehydrogenase was obtained from Boehringer and Soehne. Of a twentyfold dilution of the hexokinase, 0·02 ml was used, as well as 0·02 ml of a fiftyfold dilution of the glucose-6-phosphate dehydrogenase suspension (in 0·05 M glycylglycine; pH 7·4); these amounts were adequate to carry the reactions to completion within from 4 to 5 min at room temperature.

Hexokinase activity in *T. vulpis* was determined after homogenizing the worms in 0·05 M glycylglycine buffer (pH 7·4) at 0 °C. The homogenate was prepared by the addition of 0·2 ml of buffer per 10 mg of tissue. The same reaction mixture as the one described in the preceding paragraph was used, except that NaF (final concentration: 0·05 M) and *both* glucose and ATP (final concentrations: 0·003 M and 0·0015 M, respectively) were included, and addition of the crystalline hexokinase preparation was omitted. Immediately after homogenization, 0·02 ml of the suspension or of a dilution thereof was added to start the reaction and the increase in the optical density due to the reduction of TPN was followed at a wavelength of 340 mμ for 4 min every 30 sec. Hexokinase activity was proportional to the amount of homogenate in the reaction mixture and was linear with time.

The potassium salt of ATP was obtained from Pabst Laboratories and TPN from Sigma Chemical Corporation. Dithiazanine (3:3′-diethylthiadicarbocyanineethyl sulfate) was supplied by Dr. Koert Gerzon of Eli Lilly Laboratories and 1-ethyl-3:6-dimethyl-2-phenyl-4-pyrimido-2-cyanine chloride (CC no. 863) by Dr. L. S. G. Brooker of Eastman Kodak Research Laboratories.

RESULTS

Carbohydrate utilization

In an atmosphere of nitrogen, uptake of exogenous glucose by *T. vulpis* varied between 0·35 and 0·55 μmoles per 24 hr and per mg. (wet weight). This rate of uptake is approximately one-tenth to one-twelfth of that of *Schistosoma mansoni*[9] and one-fifth of that of *Litomosoides carinii*.[8] In the absence of added glucose, the glycogen content of *T. vulpis* decreased at a rate which was about one-half the rate of glucose uptake. These values were obtained by the determination of the rate at which glycogen disappeared over a period of 4 hr. If the parasites were incubated without glucose for longer periods they gradually lost their motility, and survival was reduced to less than 1 day. In the absence of glucose the average rate of anaerobic glycogen utilization per mg wet wt. was 0·039 μmoles (in terms of glucose units) in 4 hr, as compared to from 0·058 to 0·089 μmoles of exogenous glucose uptake during the same period. There was no difference between the rates of anaerobic and aerobic glycogen utilization. The average intial glycogen content of the worms (wet weight) varied between 2·11 and 2·62 per cent (average: 2·35). After incubation for 4 hr (37 °C) in the absence of glucose the glycogen concentration fell to between 1·53 and 1·75 per cent (average: 1·62) under anerobic, and to between 1·50 and 1·82 (average: 1·69) under aerobic conditions.

Table 1. Carbon balance of anaerobic glucose metabolism of *Trichuris vulpis*

Atmosphere	100% N_2		5% CO_2, 95% N_2		5% CO_2, 95% N_2 0·1 μg dithiazanine/ml	
	μ moles*	% of glucose-carbon taken up	μ moles*	% of glucose-carbon taken up	μ moles*	% of glucose-carbon taken up
Glucose uptake	0·41	100	0·64	100	0·36	100
Products:						
Formic acid	0·025	1·0	0·032	0·8	0·012	0·6
Acetic acid	0·103	8·3	0·175	9·1	0·084	7·9
Propionic acid	0·112	13·8	0·181	14·2	0·091	12·7
Butyric acid	0·011	1·8	0·013	1·4	0·002	0·4
n-Valeric acid	0·045	9·2	0·076	9·9	0·034	8·0
C_6 acid (s)	0·015	3·7	0·024	3·7	0·007	2·0
Lactic acid	0·054	6·7	0·074	5·8	0·082	11·7
CO_2	0·25	10·2	0·25†	6·5	0·25†	11·0
Total		54·7		51·4		54·3

* Per mg (wet weight) and 24 hr.
† Estimated on the basis of CO_2 production determined in 100 per cent nitrogen.

Products of carbohydrate metabolism

Lactic acid production accounted for no more than from 5 to 10 per cent of the carbohydrate utilized under aerobic or anaerobic conditions. Other metabolic products formed by this parasite were acetic, propionic and *n*-valeric acids (Table 1). Smaller quantities of formic, *n*-butyric and of an as yet unidentified hexanoic acid also were produced. Approximately 10 per cent of the glucose metabolized under anaerobic conditions was converted to CO_2, indicating the occurrence of active

decarboxylation mechanisms. The above products accounted for approximately 50 per cent of the carbohydrate utilized by this parasite.

Effect of carbon dioxide on carbohydrate metabolism

When *T. vulpis* was incubated in the basic medium for several days in an atmosphere of air or of nitrogen, the rate of glucose uptake was not maintained at a constant level,

TABLE 2. EFFECT OF GASEOUS ATMOSPHERE ON GLUCOSE UPTAKE OF *Trichuris vulpis*

Gas	μ moles of glucose taken up in 24 hr, (per mg wet weight)		
	1st day	2nd day	3rd day
100% N_2	0·47 0·38	0·41 0·42	0·33 0·25
Air	0·42 0·55	0·36 0·56	0·29 0·41
100% O_2	0·48 0·32	0·17 0·26	0·04 0·16
5% CO_2 95% N_2	0·75 0·87	0·80 0·92	0·78 0·88
5% CO_2 95% O_2	0·46 0·68	0·22 0·31	0·16 0·24
5% CO_2 20% O_2 75% N_2	0·68 0·76	0·38 0·22	0·26 0·14

TABLE 3. EFFECT OF THE CO_2 CONCENTRATION ON THE ANAEROBIC UPTAKE OF GLUCOSE BY *Trichuris vulpis*

Concentration of CO_2 (in N_2)	μ moles of glucose taken up in 24 hr (per mg wet weight)		
	1st day	2nd day	3rd day
—	0·38 0·40	0·33 0·13	0·31 0·09
0·1	0·48 0·36	0·19 0·33	0·07 0·30
0·3	0·43 0·36	0·31 0·29	0·35 0·25
2	0·76 0·88	0·85 0·84	0·82 0·87
5	0·71 0·92 0·86	0·76 0·82 0·86	0·71 0·86 0·80

but decreased more or less markedly (Table 2). Under these conditions another irregularity was noted: the reduction in glucose uptake was not uniform even among worms originating from the same host. On the other hand, if these *in vitro* experiments were carried out in an atmosphere more closely approaching that prevailing in the

natural habitat of the parasite,[26–30] i.e. in a gas mixture containing 5 per cent CO_2 and 95 per cent N_2, the rate of glucose uptake not only was higher than in oxygen, in air, or in various mixtures containing CO_2 and O_2, but also remained at a constant level for a period of at least 3 days (Table 2). The minimal concentration of CO_2 in nitrogen required to produce this effect was found to lie between 0·3 and 2 per cent (Table 3). The increase in the rate of glucose uptake produced by CO_2 under anaerobic conditions was demonstrable already during shorter periods of incubation lasting only 4 hr. Elimination of metabolically produced CO_2 from the atmosphere by the use of alkali resulted in a further reduction in glucose utilization (Table 4). While CO_2 increased the production of lactic and of volatile acids from glucose, the ratios of these acids formed per mole of glucose taken up were not affected materially by the presence of CO_2 in the atmosphere (Table 1).

Table 4. Effect of CO_2 on anaerobic glucose uptake of *Trichuris vulpis* during an incubation period of 4 hr

Gas	μ moles of glucose taken up in 4 hr (per mg)
N_2*	0·070
	0·091
N_2	0·110
	0·117
5% CO_2, 95% N_2	0·162
	0·146

* Center cup of incubation vessel contained 0·1 ml of 40 per cent KOH in order to absorb the CO_2 produced in the closed system.

Table 5. Survival of *Trichuris vulpis in vitro*

Medium	Survival in days			
	In air		in 5% CO_2, 95% N_2	
	Average	Limits	Average	Limits
Complete basic medium	6·0	4–7	16·2	13–17
Without potassium	4·2	3–6	6·8	2–10
Without calcium	3·2	2–4	8·6	7–12
Without magnesium	3·1	2–5	11·2	2–11
Without phosphate			12·8	7–17

Survival of T. vulpis in vitro

In an atmosphere of air the worms survived for a period of from 4 to 7 days in the basic medium.

The concentrations of the constituents of the basic medium were arrived at by varying the concentration of each cation until the optimal survival period was obtained. Omission of K^+, Ca^{2+} or Mg^{2+} resulted in a significant reduction of survival (Table 5).

In the absence of glucose, survival was reduced to less than 1 day. Glucose could be replaced by mannose, fructose, maltose or glycerol (Table 6), indicating that one or

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several hexokinases, a glycerol kinase, a maltase and a phosphomannose isomerase were present in the worms. On the other hand, galactose, sucrose, trehalose and ribose did not support the survival of the worms (Table 6).

When air was replaced by a gas mixture containing 5 per cent CO_2 and 95 per cent nitrogen, survival of the worms was increased significantly (Table 7). As in the experiments concerned with glucose uptake, this effect of CO_2 required the absence of oxygen. When survival was extended to a period of 2 weeks by the use of the CO_2–N_2 gas mixture it became evident that glucose was more effective in supporting the survival of *T. vulpis in vitro* than were mannose, fructose, maltose or glycerol (Table 6). Under these conditions, the requirement of the worms for K^+, Ca^{2+} and Mg^{2+} was demonstrable also (Table 5).

TABLE 6. EFFECT OF VARIOUS SUGARS ON THE SURVIVAL OF *Trichuris vulpis in vitro*

Sugar	Concentration (mg/ml)	Survival in days			
		In air		in 5% CO_2, 95% N_2	
		Average	Limits	Average	Limits
—	—	0	0	0	0
Glucose	2	6·0	5–7	14·8	14–16
Fructose	2	5·6	5–6	7·2	5–8
Mannose	2	5·4	5–6	10·3	9–11
Glycerol	2	4·2	3–6	5·3	5–6
Galactose	2	0·4	0–1	—	—
Maltose	4	4·4	2–6	4·0	2–5
Sucrose	4	0	0	—	—
Trehalose	4	0	0	—	—
Ribose	2	0·2	0–1	—	—

TABLE 7. EFFECT OF THE GASEOUS ATMOSPHERE ON THE SURVIVAL OF *T. vulpis in vitro* 37 °C

Gas	Survival (days)	
	Average	Limits
Air	6·0	4–7
5% CO_2 in air	5·2	2–9
N_2	6·0	4–9
5% CO_2 in N_2	11·2	9–16

Respiratory enzymes

During incubation of *T. vulpis* in the basic medium with air as the atmosphere, the parasites took up 0·34 μmoles of oxygen per mg (wet weight) in 24 hr (37 °C). Evidence for the presence of the cytochrome system in *T. vulpis* was supplied by the observations summarized in Table 8. Homogenates of the worms catalyzed the reduction of cytochrome *c* and this rate was increased significantly by the addition of succinate. Furthermore, reduced cytochrome *c* was oxidized by homogenates of the worms and this reaction was sensitive to cyanide and heat. These observations suggest that the worms contain a succinoxidase which operates with the participation of the cytochrome system. The activities of both cytochrome *c* and of cytochrome oxidase in homogenates are sufficiently high to account for the rate of oxygen uptake of intact worms.

Addition of hydrogen peroxide to homogenates of *T. vulpis* resulted in the rapid release of oxygen (Table 8) indicating that these worms have high catalase activity.

Concentrated aqueous extracts of *T. vulpis* have a distinct red coloration. Using a cytochrome *c*-deficient rat liver succinoxidase preparation[31] it was found that this pigment was not identical with cytochrome *c*. The pigment had a maximum extinction

TABLE 8. SUCCINIC DEHYDROGENASE, CYTOCHROME OXIDASE AND CATALASE ACTIVITIES OF *Trichuris* HOMOGENATES

Exp. no.	Reaction	Measured component	Rate
			mμmoles/hr per mg
1	Endogenous ⟶ 2 cytochrome *c*	Cytochrome *c*	5·8
2	succinate ⟶ 2 cytochrome *c*	Cytochrome *c*	12·7
3	2 Cytochrome *c* ⟶ O_2	Cytochrome *c*	12·4
4	2 Cytochrome *c* ⟶ O_2 + KCN	Cytochrome *c*	4·7
5	2 H_2O_2 ⟶ 2 H_2O + O_2	O_2	229·0

Reaction mixtures in experiment nos. 1 to 4 contained 50 μmoles of phosphate buffer (pH 7·7), 0·04 μmoles of reduced or 0·05 μmoles of oxidized cytochrome *c*, 0·02 ml *Trichuris* homogenate and, where indicated, 1 μmole of KCN or 50 μmoles of sodium succinate, or both. Final volume: 1·0 ml. The homogenate was prepared in sucrose (20 per cent) containing 0·01 M phosphate buffer (pH 7·7) and 68 mg of tissue per ml. Measurements were taken in the Beckman DU spectrophotometer at 550 mμ at room temperature (25 °C). Experiment number 5 was carried out in the presence of 40 μmoles of phosphate buffer (pH 7·3), 3·0 μmoles of H_2O_2 and 0·2 ml of *Trichuris* homogenate. Controls were run in the presence and absence of homogenate and of H_2O_2. The *Trichuris* homogenate was prepared in sucrose (20 per cent) containing 0·05 M phosphate buffer (pH 7·3) and contained 121 mg of tissue per ml. Gas production was determined in the Warburg apparatus (37 °C) with 0·1 ml of 10 N KOH in the center well.

at a wavelength of 414 mμ, which was shifted to 426 mμ on reduction with a few crystals of sodium hydrosulfite. This behavior is more characteristic for a hemoglobin rather than for a cytochrome. Possibly this pigment originates from the red cells of the host because it has been reported that trichurids secure their food from mucosal tissue and blood.[32–35]

Effect of dithiazanine on metabolism of T. vulpis

Concentrations of dithiazanine lower than those which affected the motilityof the worms significantly inhibited their rate of anaerobic glucose uptake (Table 9). Therefore it appears that dithiazanine has a direct effect upon the carbohydrate metabolism of *T. vulpis* and that these biochemical changes are not secondary to a reduction in the motility of the worm. When the organisms were exposed for 24 hr to from 0·1 to 0·2 μg of the drug per ml and were then transferred into dithiazanine-free medium, the inhibition of glucose uptake persisted, and became even more pronounced in many experiments (Table 9). This metabolic effect of dithiazanine was found to be irreversible. The inhibitory effects of dithiazanine on glucose uptake were equally pronounced in the presence and in the absence of CO_2. Reduction of glucose uptake by dithiazanine was associated with a corresponding decrease in the amount of volatile acids produced, but there was no specific inhibition of the production of any particular volatile acid (Table 1).

Exposure of the worms to concentrations of dithiazanine which produced a reduction in glucose uptake resulted in a marked decrease in the concentrations of free glucose, of ATP and of total carbohydrate in the worms (Table 10).

Dithiazanine had no effect on the hexokinase activity of homogenates of *T. vulpis*. The highest drug concentration used was 5 μg/ml which is fifty times higher than the one which produced decreases in glucose uptake and in the glucose concentration in the worm.

Uptake of glucose by *T. vulpis* and the concentration of free glucose in the nematode were decreased also by another cyanine dye, 1-ethyl-3:6-dimethyl-2-phenyl-4-pyrimido-2-cyanine chloride. The inhibitory effects of this dye were much weaker and considerably higher concentrations were required. Furthermore, in contrast to dithiazanine, the actions of this cyanine dye were reversible.

TABLE 9. EFFECT OF DITHIAZANINE ON GLUCOSE UPTAKE BY *Trichuris vulpis*
(Atmosphere: 5% CO_2, 95% N_2, 37 °C)

Dithiazanine concentration during first day* (μg/ml)	Glucose uptake (μ moles†)		
	1st day	2nd day	3rd day
—	0·66	0·63	0·61
—	0·77	0·65	0·69
—	0·68	0·65	0·68
0·1	0·60	0·42	0·46
0·1	0·48	0·35	0·33
0·1	0·55	0·35	0·28
0·15	0·42	0·38	0·30
0·15	0·56	0·26	0·22
0·2	0·48	0·36	0·27
0·2	0·40	0·22	0·16
0·2	0·44	0·29	0·14

* These worms were incubated for 24 hr in a medium containing dithiazanine and then transferred to the drug-free basic medium which was used throughout for the control worms.

† Per mg worm wet wt.

DISCUSSION

Since *T. vulpis* survives *in vitro* for considerably longer periods of time in an atmosphere of 5 per cent CO_2 in nitrogen than in one containing the same concentration of CO_2 in air or oxygen, it is evident that the energy required by *T. vulpis* is provided by anaerobic, rather than by aerobic, metabolism. The possibility that hydrogen peroxide produced during respiration may have a deleterious effect can be ruled out because catalase activity of the worms is far in excess of the rate of oxygen uptake. Therefore, even if all dehydrogenation reactions were mediated by a terminal electron acceptor catalyzing the production of hydrogen peroxide, the latter would be decomposed as soon as it was formed. It is doubtful whether hydrogen peroxide is a respiratory product of *T. vulpis* formed by a terminal flavoprotein, because cytochrome *c* and cytochrome oxidase activities are sufficiently high to mediate the respiration of the worm. The presence of the cytochrome system and of a cytochrome *c* linked succinoxidase

system distinguishes *T. vulpis* from two other nematodes, *Ascaris lumbricoides* and *Litomosoides carinii*, in which the cytochrome system could not be detected,[36] and from *Schistosoma mansoni* in which cytochrome *c* and cytochrome oxidase activities account for less than 10 per cent of the oxygen taken up by this trematode.[36] Since the habitat and metabolism of the adult whipworm are essentially anaerobic, the cytochrome system in this organism may play no physiological role, but may have been carried over from a previous, aerobic, stage in the life cycle of the nematode.

Table 10. Effect of dithiazanine on the concentrations of glucose, ATP and total carbohydrate in *Trichuris vulpis*
(Atmosphere: 5% CO_2, 95% N_2, 37 °C)

Exp. no.	Incubation period (hr)	Dithiazanine (μg/ml)	Glucose (μmoles*)	ATP (μmoles*)	Total carbohydrate (μmoles*)
1	24	—	5·52	1·55	125
	24	0·1	1·46	0·89	64
2	24	—	8·70	1·53	146
	24	0·1	1·73	0·76	78
3	48	—	7·30	1·50	167
	48	0·1	1·58	0·48	36
4	48	—	6·67	1·66	152
	48	0·1	1·88	0·58	43
5	72	—	8·35	1·76	185
	72	0·1	0·92	0·36	41

For the first 24 hr the worms were incubated in a dithiazanine containing medium. At the end of this period they were transferred into a dithiazanine-free medium and incubated for another 24 (experiments 3 and 4) or 48 (experiment 5) hrs.
* Per g wet wt.

The marked effect of CO_2, in increasing the period of survival of *T. vulpis*, *in vitro*, and in enhancing, as well as maintaining, the rate of glucose uptake, may be due to mechanisms for CO_2 fixation which are essential to the functional integrity of the worm. The high CO_2 tension prevailing in the intestinal tract and contents of the host[26–30] insures the availability of this gas to the parasite in its natural habitat. CO_2 is known to be a growth factor for some micro-organisms.[37–43] Furthermore, highly active systems for CO_2 fixation have been demonstrated in two intestinal nematodes, *Heterakis gallinae*[44] and *Ascaris lumbricoides*.[45] In these organisms CO_2 condenses with pyruvate eventually giving rise to succinate. Unpublished observations of two of the authors (E.K. and E.B.) have indicated that from 10 to 15 per cent of the carbohydrate utilized by *T. vulpis* is converted to succinate. Should this be confirmed, CO_2 fixation may be prevalent in this nematode also. In addition, propionate is a fermentation product of *T. vulpis*. It has been demonstrated that in muscle strips of *Ascaris*, propionate is formed by the decarboxylation of succinate.[45] Should a similar reaction account for the formation of propionate by *T. vulpis*, at least 25 per cent of the carbohydrate utilized by this parasite would be metabolized through pathways involving CO_2 fixation.

An inhibition of glucose uptake by *T. vulpis* was observed with concentrations of dithiazanine which produced no change in the motility of the worms. Therefore this

biochemical effect is not secondary to, but precedes the anthelmintic action of the cyanine dye. If this decrease in the uptake of glucose was due to an inhibition of one or of several enzymes concerned with the intracellular metabolism of carbohydrate there should be an accumulation of glucose within the worm. However, the reduction of glucose uptake produced by dithiazanine was associated with a marked decrease in the concentration of free glucose in the worm. Therefore it is apparent that dithiazanine interferes with the uptake of glucose from the medium, rather than with its utilization. Reduced availability of exogenous glucose due to an inhibition of the transport of this sugar into the parasite should increase the utilization of endogenous carbohydrate reserves. This is consistent with the marked decrease in the concentration of the total carbohydrate of the worms which had been incubated with dithiazanine, indicating again that dithiazanine did not interfere with the intracellular utilization of carbohydrate. Furthermore, even high concentrations of dithiazanine had no inhibitory effect on the activity of hexokinase in homogenates of *T. vulpis*; therefore, the cyanine dye did not inhibit the phosphorylation of glucose present in the parasite. It is concluded that the depletion of the carbohydrate reserves is secondary to the decreased availability of exogenous glucose due to interference by dithiazanine with the transport of this sugar into the worm. This circumstance, in turn, leads to a decrease in the rate at which energy-rich phosphate bonds are formed, and accounts for the reduction in the concentration of ATP in the parasite. Owing to these changes in carbohydrate metabolism brought about by dithiazanine, the supply of energy required for survival is decreased and eventually should become inadequate, resulting in the death of the worm.

The biochemical effects of dithiazanine on *T. vulpis* are irreversible, since they persist and become even more pronounced after exposure of the worms to the cyanine dye has been discontinued by transfer into dithiazanine-free medium. This property is in agreement with the observed irreversibility of the chemotherapeutic action on *Trichuris trichiura* after oral administration of the drug.[1]

It is not known whether glucose enters *T. vulpis* through the cuticle or via the alimentary canal. In the latter eventuality the possibility should be considered that dithiazanine interferes with the intestinal absorption of glucose.

The biochemical effects of another cyanine dye (CC no. 863) qualitatively were similar to those of dithiazanine, but, higher concentrations were required to obtain these effects, which proved to be reversible in the case of this particular dye. The latter cyanine is one of the most potent ones with regards to its ability to inhibit oxygen uptake of *L. carinii*.[7, 8] Thus, there is no parallelism between these two types of effects of cyanines on helminths; in fact, two unrelated mechanisms may be involved. It remains to be determined which particular structural characteristics confer to a cyanine dye high activity and irreversibility for the inhibition of glucose transport in *T. vulpis*. Furthermore, the question arises whether there exists a relationship between the inhibitory effects of cyanines on glucose transport in *T. vulpis* and those on the active transport of organic bases across renal tubuli reported by Peters *et al.*[46–48]

Among helminths, considerable differences in biochemical characteristics have been encountered, even among species which are closely related to each other morphologically and taxonomically.[17, 18] Although *T. trichiura*, the whipworm invading man, and *T. vulpis* are equally susceptible to the chemotherapeutic actions of dithiazanine,[1, 49] investigations of the effects of this cyanine dye on *T. trichiura* are required

in order to ascertain more definitely whether inhibition of glucose transport accounts for the anthelmintic action of dithiazanine in human trichuriasis.

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STUDIES ON THE GABA PATHWAY—I

THE INHIBITION OF γ-AMINOBUTYRIC ACID–α-KETOGLUTARIC ACID TRANSAMINASE *IN VITRO* AND *IN VIVO* BY U-7524 (AMINO-OXYACETIC ACID)

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(*Received* 15 *July* 1960)

Abstract—(1) Amino-oxyacetic acid has been found to be a potent inhibitor of the enzyme γ-aminobutyric acid-α-ketoglutaric acid transaminase derived both from *E. coli* and mammalian brain.

(2) The nature of this inhibition has been demonstrated to be strictly competitive.

(3) Amino-oxyacetic acid has also been demonstrated to inhibit the transaminase in the brains of several species of animals causing marked elevations in the brain concentrations of γ-aminobutyric acid.

(4) An assay method for γ-aminobutyric acid was developed using naturally occurring γ-aminobutyric acid-α-ketoglutaric acid transaminase and succinic semialdehyde dehydrogenase derived from *E. coli*.

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THERE is abundant evidence extant[1–3] that γ-aminobutyric acid (GABA) is found in remarkably high concentrations in the central nervous system and that it may have an important physiological role in the transmission of certain types of nervous impulses. Administration of GABA in massive intravenous doses does not cause an elevation in brain concentrations of this amino acid, presumably because GABA does not cross the blood–brain barrier in appreciable quantities. Thus, it has been difficult to elucidate the effects of increased concentrations of GABA in the brains of intact animals. Roberts and Frankel[4, 5] demonstrated that GABA is formed in brain by the decarboxylation of glutamic acid. Subsequently, as shown by Bessman *et al.*[6] and Roberts and Bregoff[7], GABA transaminates with α-ketoglutaric acid (KGA) to form glutamate and succinic semialdehyde. As this is the principal pathway for the degradation of this amino acid in brain, inhibition of this transamination step should result in increased brain concentrations of GABA. Baxter and Roberts[8] have reported that such an inhibition can be induced with suitable doses of hydroxylamine, approximately doubling the concentration of GABA in the brain of rats.

This report is concerned with the enzymic and some of the physiological properties of a much more potent inhibitor of this enzyme, namely amino-oxyacetic acid (AOAA) ($H_2N.O.CH_2.COOH$) and its effects on the levels of GABA in the brain in various species of animals.

EXPERIMENTAL

Material and methods

When this study was initiated it was realized that the GABA–KGA transaminase found in brain of most species is not a very active enzyme compared to the same transaminase from bacterial sources. It is also more labile to freezing and thawing. When the brain transaminase is isolated from acetone powders it is usually contaminated with free α-amino acids which chelate copper. These interfere with the copper chelation method of Baxter and Roberts[9], which is the most rapid method currently available for the estimation of glutamic acid. Thus, it was felt that the brain enzyme was a poor one to employ in testing potential inhibitors. Baxter and Roberts[9] reported that *E. coli*, strain E-26 had a very active GABA–KGA transaminase. An investigation of this and other strains of *E. coli* showed that a number of them had the required enzyme and one strain, ATCC-26, when grown in nutrient broth (Difco), was superior to other strains for this transaminase activity. This organism was also found to have a very active succinic semialdehyde dehydrogenase. Jakoby and Scott[10] described a coupled system in which these two enzymes, isolated as adaptive enzymes from *Pseudomonas fluorescens* ATCC 13,430, grown on a synthetic medium rich in pyrollidine, could be used in an assay for either GABA or KGA by following the reduction of TPN at 340 mμ. The naturally occurring enzymes in *E. coli* ATCC-26, when isolated in the manner to be described, can be used either as a coupled system for the assay of GABA, or for transaminase activity alone, when testing the activity *in vitro* of potential inhibitors.

The organism is grown on 10-ml slants of nutrient agar (Difco) and is transferred monthly to fresh slants. When a batch of cells is to be grown, 6 l. of nutrient broth (Difco) are divided into 150-ml aliquots in 500-ml shake flasks and autoclaved. When cool, the flasks are seeded with a suspension of organisms from a slant and are then shaken at 250 rev/min at from 28 to 29 °C for from 16 to 18 hr. Good growth should be evident at this time. The cells are harvested by centrifugation and washed twice in at least 10 vols. of cold saline, after which they are frozen. The usual yield of cells is from 17 to 18 g. When frozen, the cells may be stored indefinitely without loss of enzymic activity.

When desired, the cells are thawed and washed once in 10 vols. of 0·1 M potassium phosphate buffer, pH 7·35, containing 0·01 per cent 2-mercaptoethanol. The cells are then resuspended in 1 vol. of this buffer and are treated for 30 min at from 0 to 3 °C in a Raytheon sonic oscillator.* The preparation is centrifuged for 20 min at 12,000 *g* in order to remove cellular debris and unbroken cells. The supernatant fluid is then adjusted to a protein concentration of from 65 to 75 mg/ml and is designated E_0. This crude extract has contaminating enzymes which react with compounds in brain other than GABA to cause a reduction of TPN. This makes accurate assays impossible without further purification.

In the fractionation scheme to be described, all volumes refer to the initial volume of sonicate taken for fractionation. To 15 ml of the crude sonicate, 0·05 vols. of 1 M $MnCl_2$ are added dropwise and with good mixing. The precipitate of nucleic acid which forms is removed by centrifugation and discarded. To the supernatant solution are added dropwise and with rapid mixing 0·8 vol. of cold saturated ammonium sulfate

* The machine used is rated at 200 W and 10 kc. The power supply was set at a current output of 1·0 A.

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solution adjusted to a pH of 7·4 (measured at a 1:10 dilution in water at room temperature). The precipitate is removed by centrifugation and discarded. To the supernatant fluid is added 1 vol. of the ammonium sulfate solution (pH 7·4). The precipitate is collected by centrifugation and dissolved in 1 vol. of 0·1 M potassium phosphate buffer, pH 7·35, containing 0·01 per cent 2-mercaptoethanol. This fraction contains the desired enzymes. The yield is from 60 to 70 per cent of the initial activity of the sonicate, with a 1·5- to 2-fold purification.

The components of the mixture used in the determination of enzyme activity which are added to a Beckman DU cuvette, are as follows: 6 μmoles of GABA; 5 μmoles of 2-mercaptoethanol; 6 μmoles of α-ketoglutaric acid; 600 μmoles of tris buffer, pH 8·35; 1 mg of TPN; enzyme; water to a final volume of 3 ml. (An enzyme unit is arbitrarily defined as that amount of enzyme which will induce an OD change of 0·001 unit per min. under specified conditions of assay.)

The contents of the cell are well mixed and a reading at 340 mμ is immediately taken against a blank containing all the components except GABA. Readings are taken at 1-min intervals for 5 min at room temperature. Over this period of time, with limiting amounts of enzyme, the reaction is linear and zero order. The reading taken at zero time is subtracted from the reading taken at 5 min, and the corrected reading divided by 5 indicates the units of OD change per minute. Calculations of specific activity of the enzyme, total units, and per cent recovery may then be made. As isolated, the purified preparation contains from 18 to 20 mg of protein and approximately from 2500 to 3000 units per ml. One-tenth of a milliliter of this preparation is more than adequate to assay samples under the specified conditions within a range of concentration of from 0·1 to 0·6 μmoles of GABA. On known GABA standards made up in ethanol–water (75:25, v/v), the error of the assay is less than 3 per cent. The method is sensitive to 0·05 μmoles of GABA.

This preparation has a pH optimum of 8·2 to 8·4. The specificity of the system has not been extensively studied, but there is no reduction of TPN when glycine, ornithine, β-alanine, butyronitrile, 4-methylaminobutyric acid, or 2-hydroxy-4-aminobutyric acid are added. Tissues other than brain do contain one or more compounds which are extractable with the aqueous ethanol and which cause a slight reduction of TPN in the system. Chromatographic examination of these extracts has not revealed the presence of GABA, and the concentrations of these compounds remain unaffected by doses of AOAA which cause from four- to five-fold increases in the amounts of GABA in the brain.

The preparation is stable when frozen and may be kept in this state for at least a month without appreciable loss of activity. With multiple freezing and thawing, however, there may be some loss of activity and it is well to run a calibration curve on known standards before attempting to use the preparation for tissue assays.

Brain samples are prepared for analysis essentially in the manner described by Roberts and Frankel[1]. Small animals are decapitated and large animals are sacrificed by intravenous injections of magnesium sulfate. The brains are rapidly removed and frozen in dry ice, after which they are blended in 10 vols. of ethanol–water (75:25) in a micro-head of a Waring blendor. One minute is usually sufficient for from 1 to 3 g of tissue. The homogenate is centrifuged at 5000 *g* and a 10-ml aliquot of the clear supernatant fluid is then dried *in vacuo*. The residues, when dissolved in 0·1 vol. of the aqeuous ethanol, are ready for assay. Frequently it is not possible to dissolve all of

the dry residue, but it appears that all the GABA present in the sample goes readily into solution. Of this solution, the usual aliquot which is added to the cuvettes for analysis is 0·05 ml.

It must be pointed out that the enzyme preparation is contaminated with a small amount of alcohol dehydrogenase. Also, the system is inhibited to some extent by ethanol. To compensate for this it is necessary to include in the blank the same volume of the aqueous ethanol as is added with the brain samples. The components added to the cuvettes for tissue determinations are the same as given for the estimation of enzyme activity, with the obvious omission of GABA.

While the system described could be used for screening compounds, the discovery of an inhibition would require the demonstration that the transaminase and not the dehydrogenase was the affected enzyme. For this reason the preparation was used for its transaminase activity alone when testing potential inhibitors. The test system which is the same as that used by Baxter and Roberts[9], is composed in the following manner. Into 15-ml conical glass centrifuge tubes are added: 40 μmoles of GABA; 40 μmoles of KGA; 50 μmoles of sodium borate buffer, pH 8·2; enzyme sufficient to form from 3 to 4 μmoles of glutamate per hour; water to a final volume of 1·5 ml.

The test compounds were made up in 0·2 M sodium borate buffer, pH 8·2, at a concentration of 0·1 M, and 0·1 ml was added to the tubes. Appropriate enzyme and substrate blanks were included, as well as blanks for each compound tested. The contents of the tubes were well mixed and placed in a water bath at 38 °C for 30 min, after which they were removed from the bath and chilled. The reaction was stopped by the addition of 0·5 ml of 25 per cent trichloroacetic acid. The analysis for glutamic acid was carried out in the manner described by Baxter and Roberts[9].

When an inhibitor of the transaminase was found it was titrated in decreasing concentrations to determine its potency. Confirmation of inhibition was obtained from chromatograms developed in a descending front with phenol–water (88:12) as the developing solvent. The strips were usually left in the tanks for a 24-hr period and then dried in an oven at 80° C for 1 hr. The strips were sprayed with a 1 per cent solution of ninhydrin in butanol and again heated for 30 min in the oven to develop the color.

When a promising inhibitor was disclosed with the *E. coli* transaminase, it was administered intravenously to rats at several different doses. The intravenous route was used to obviate any difficulties which might be encountered with absorption. The rats were then held for a minimum of 4 hr before they were sacrificed for determinations of GABA in the brain.

RESULTS

Amino-oxyacetic acid (AOAA) was found to inhibit the bacterial transaminase 100 per cent at a concentration of $3{\cdot}3 \times 10^{-4}$ M and 40 per cent at a concentration of $3{\cdot}3 \times 10^{-6}$ M. It has no effect on the bacterial succinic semialdehyde dehydrogenase at concentrations of $3{\cdot}3 \times 10^{-2}$ M.

When the brain transaminase was tested under similar conditions, it was inhibited 92 per cent at a concentration of AOAA of 1×10^{-5} M.

When AOAA was tested in rats, in the manner described, it was apparent that the compound, in spite of being a strong acid and completely ionized at physiological pH levels, crossed the blood–brain barrier in amounts sufficient effectively to inhibit

brain GABA–KGA transaminase. This was reflected in the increased concentrations of GABA which were observed. In Table 1 are shown the brain GABA levels which resulted from administration of AOAA at various doses in five species of animals. While there was a definite accumulation of GABA in all species, the dog proved to be most refractory and unpredictable in its response. The guinea pig, while not extensively studied, was most sensitive to the toxic effects of AOAA.

TABLE 1. DOSE OF AMINO-OXYACETIC ACID AND RESULTING GABA LEVELS IN FIVE SPECIES OF ANIMALS

Species	Dose of AOAA (mg/kg)	Route of administration	Interval before sacrifice (hr)	GABA in brain (μmoles/g)	Number of animals/group
Rat	none	—	—	2·5	2
Rat	6·25	i.v.	5·5	2·5	2
Rat	12·5	i.v.	5·5	5·2	2
Rat	25	i.v.	5·5	6·8	2
Rat	50	i.v.	5·5	10·0	2
Rat	none	—	—	4·4	2
Rat	25	oral	6	3·7	2
Rat	50	oral	6	4·3	2
Rat	100	oral	6	4·7	2
Rat	200	oral	6	9·0	2
Rat	6·25	subcut.	6	5·6	2
Rat	12·5	subcut.	6	7·6	2
Rat	25	subcut.	6	10·5	2
Rat	50	subcut.	6	10·8	2
Mouse	none	—	—	2·7	5
Mouse	6·25	subcut.	6	7·3	5
Mouse	12·5	subcut.	6	8·6	5
Mouse	25	subcut.	6	13·0	5
Mouse	50	subcut.	6	12·2	5
Cat	none	—	—	1·9	1
Cat	10	subcut.	6	3·0	1
Cat	20	subcut.	6	5·6	1
Cat	30	subcut.	6	10·2	1
Guinea pig	none	—	—	2·6	2
Guinea pig	10	subcut.	6	7·7	2
Guinea pig	20	subcut.	6	9·2	2
Dog	none	subcut.	4	1·7	1
Dog	50	subcut.	4	2·0	1
Dog	100	subcut.	4	5·0	1

As shown in Table 2, the peak levels of GABA which were reached in from 6 to 8 hr were followed by a slow decline. Twelve hours after administration of the inhibitor the brain levels in the rat, mouse, cat, and guinea pig were markedly elevated, as compared to those of the controls. Even 24 hr after dosage with AOAA, there was still some elevation of GABA in these species.

Gross physical effects seen following administration of AOAA were qualitatively similar in all species studied. In those animals able to do so, i.e. the cat and dog, there was usually some emesis; this occurred shortly after either oral or parenteral

administration. Then there ensued a gradual sedation and paralysis of the skeletal musculature. There was no marked analgesia or anesthesia as the animals, except when under very high doses, could be aroused. The palpebral, corneal, pinna and nasal reflexes were present even when the animals were very markedly sedated. These effects persisted for 12 hr or more, after which the animals returned to normal. All gross signs of the toxicity of AOAA disappeared within 24 hr.

TABLE 2. TIME CURVES OF GABA LEVELS FOLLOWING THE ADMINISTRATION OF AMINOOXYACETIC ACID IN FIVE SPECIES OF ANIMALS

Species	Dose of (mg/kg)	Route of administration	Interval before sacrifice (hr)	GABA in brain (μmoles/g)	Number of animals/group
Rat	none	i.v.	—	2·0	2
Rat	37·5	i.v.	1	3·8	2
Rat	37·5	i.v.	2	6·9	2
Rat	37·5	i.v.	4	6·4	2
Rat	37·5	i.v.	8	11·7	2
Rat	37·5	i.v.	12	9·0	2
Rat	37·5	i.v.	23	5·6	2
Rat	50	s.c.	1	3·1	2
Rat	50	s.c.	2	4·0	2
Rat	50	s.c.	4	6·2	2
Rat	50	s.c.	6	8·0	2
Rat	50	s.c.	8	7·8	2
Rat	50	s.c.	12	7·8	2
Rat	50	s.c.	24	3·8	2
Rat	50	s.c.	48	1·4	2
Mouse	none	s.c.	—	2·4	5
Mouse	30	s.c.	1	2·3	5
Mouse	30	s.c.	2	4·3	5
Mouse	30	s.c.	4	6·3	5
Mouse	30	s.c.	8	7·3	5
Mouse	30	s.c.	12	6·5	5
Mouse	30	s.c.	24	3·7	5
Cat	none	s.c.	—	1·9	1
Cat	30	s.c.	1	2·5	1
Cat	30	s.c.	2	2·7	1
Cat	30	s.c.	4	8·0	1
Cat	30	s.c.	6	10·2	1
Cat	30	s.c.	8	5·9	1
Cat	30	s.c.	12	4·7	1
Dog	none	s.c.	—	1·7	1
Dog	40	s.c.	1	1·8	1
Dog	40	s.c.	2	1·7	1
Dog	40	s.c.	3	2·3	1
Dog	40	s.c.	4	5·8	1
Dog	40	s.c.	6	6·4	1
Dog	40	s.c.	8	5·8	1
Guinea pig	25	s.c.	—	2·6	2
Guinea pig	25	s.c.	1	5·4	1
Guinea pig	25	s.c.	2	7·7	1
Guinea pig	25	s.c.	4	11·5	1
Guinea pig	25	s.c.	6	13·8	1
Guinea pig	25	s.c.	8	10·7	1
Guinea pig	25	s.c.	12	11·6	1
Guinea pig	25	s.c.	24	9·6	1

Mechanism of inhibition

As the *E. coli* transaminase had served as a valid model for the brain enzyme *in vivo*, kinetic studies were conducted with this preparation. In order to decrease the reaction rate, the pH was decreased from 8·2 to 7·4 and the temperature of the incubation was decreased to 30 °C. Under these conditions the reaction was linear and zero order over a 15-min period. The reaction was then run at seven different concentrations of GABA and four different concentrations of AOAA, in addition to a set of controls run at seven different concentrations of GABA in the absence of inhibitor. When the reciprocals of substrate concentration were plotted against reciprocals of velocity, in the manner described by Lineweaver and Burk[11] the intercept of the slopes remained constant, but the slope was increased by $(1 + I/K_i)$ with increasing concentrations of inhibitor, a finding which demonstrates that the inhibition was competitive. The calculated Michaelis–Menten constant (K_m) for GABA was $2{\cdot}76 \times 10^{-2}$, indicating a rather low affinity of the enzyme for GABA. On the other hand, the K_i for AOAA under these conditions was $7{\cdot}52 \times 10^{-6}$. The ratio between these two values is greater than 3600, which indicates that there is a rather remarkable difference between the affinity of the enzyme for the inhibitor, as opposed to its natural substrate, GABA.

These experiments were repeated at seven different concentrations of α-ketoglutaric acid and three different concentrations of AOAA, together with appropriate controls. The calculated K_m for KGA was $9{\cdot}66 \times 10^{-3}$, and the K_i under these conditions was $9{\cdot}16 \times 10^{-6}$. This value is in reasonable agreement with the preceding value of $7{\cdot}52 \times 10^{-6}$.*

DISCUSSION

Welch and Bueding[12] and Hunter and Lowry[13] in their discussions of effects of drugs on enzymes, concur on three important criteria which must be met before an enzyme inhibition observed *in vitro* may be said to occur *in vivo*. These points are: (1) there must be definitive proof that the inhibitor is selectively inhibiting the same enzyme in experimental animals, (2) in animals this inhibition must occur at approximately the same concentration of inhibitor as that observed *in vitro*, and (3) a direct relationship between increasing concentrations of inhibitor and observable effects relative to an increasing degree of inhibition of the enzyme must be demonstrable.

In this instance of brain γ-aminobutyric-α-ketoglutaric acid transaminase, these criteria have all been met. With regard to the first point, inhibition of this transaminase *in vivo* should result in increased concentrations of brain GABA. That this occurs has been demonstrated by a highly specific enzyme assay system, and also by paper chromatograms.

The second postulate is more difficult to assess, because as the inhibition is strictly competitive in nature, the degree of inhibition of the enzyme will be a function of absolute amounts of enzyme and substrates relative to concentrations of inhibitor. Under the conditions of assay used for testing compounds, in which substrate concentrations are far in excess of those found *in vivo*, and in which a transaminase preparation from cat brain is used, a 92 per cent inhibition of the enzyme was observed

* The author wishes to express his gratitude to Dr. E. R. Garrett, of the Department of Physical and Analytical Chemistry of The Upjohn Company, for his generous help in calculating these constants.

at an AOAA concentration of 1×10^{-5} M. G. R. Zins, of these laboratories, in as yet unpublished studies with ^{14}C-labelled AOAA observed concentrations of 1×10^{-5} M of this compound or its metabolites in the brains of rats within 1 hr after intravenous administration of a dose of 20 mg/kg. If the inhibitor were present entirely in unchanged form at the site of the enzyme, with the markedly lower substrate concentrations present, this level of AOAA should induce complete inhibition of the enzyme. That a very marked inhibition at this dose does in fact occur may be derived from the data of Table 1, which show that an intravenous dose of 25 mg/kg of AOAA to rats caused at least twofold increase in brain GABA.

The data of Table 1 also fulfill the third criterion by demonstrating that in several species there is an increasing accumulation of GABA with increasing doses of AOAA.

So far in this discussion only one aspect of the effects of AOAA has been dealt with, namely its influence as an inhibitor of GABA–KGA transaminase *in vivo*. When AOAA is given in toxic doses to all species of animals studied, convulsions are observed which terminate in a respiratory death. These effects cannot be attributed solely to the inhibition of GABA–KGA transaminase. Preliminary observations show that the free amino acid profile in livers of dogs is markedly altered by a convulsive dose of AOAA and thus other enzymes are probably affected. A dilemma is therefore posed as to what effects in the intact animal are caused by increasing cerebral concentrations of GABA as opposed to other and unrelated actions.

One result of inhibiting GABA–KGA transaminase, possibly of great importance, is a concomitant decrease in synthesis of succinic semialdehyde which would follow from increasing concentrations of brain GABA. As GABA and succinic semialdehyde are the only compounds unique to the GABA pathway, some of the gross effects observed in intact animals could well be attributed to this reciprocal effect. The importance of GABA in relationship to succinic semialdehyde, and the discrete role of succinic semialdehyde in brain have yet to be elucidated.

There is a marked discrepancy between the effects of hydroxylamine on GABA levels in rats, as reported by Baxter and Roberts[9], as contrasted to the effects seen with AOAA. With hydroxylamine, peak levels of GABA are observed within from 1 to 2 hr after administration compared to peak levels of GABA observed at from 6 to 8 hr with AOAA. The kinetics of inhibition by hydroxylamine have not been described but the work of Baxter and Roberts[9] is strongly suggestive that the inhibition is not competitive but is due to oxime formation between hydroxylamine and pyridoxal phosphate on the active surface of the enzyme. With mammalian GABA–KGA transaminase preparations, these authors observed that inhibition of the enzyme with hydroxylamine could be reversed by increased concentrations of pyridoxal phosphate. Inhibition of the mammalian enzyme by AOAA cannot be reversed by pyridoxal phosphate. Thus hydroxylamine and AOAA apparently inhibit the enzyme by two different mechanisms, which may be the reason why the results on GABA levels are different.

The observations presented in this report delineate one important physiological property of GABA. It has not been possible to demonstrate GABA in the cerebrospinal fluid of normal dogs or of dogs treated with AOAA, in which it was possible to show a markedly elevated brain level of GABA. It appears, therefore, that GABA is confined to the brain cells, because if appreciable quantities left the cells, one would expect the amino acid to appear in the cerebrospinal fluid. These results also suggest

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that GABA is not utilized at extracellular sites. Inhibition of the transaminase should favor other reactions in which GABA may be involved, but the very high levels of GABA which are attained in most species following inhibition of the transaminase, and the length of time during which these concentrations are maintained, point to the conclusion that the transaminase reaction and subsequent pathway are the principal route for the utilization of GABA.

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SPECIFICITY OF SUBSTITUTED PHENYL PHOSPHORUS COMPOUNDS FOR ESTERASE INHIBITION IN MICE

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(*Received* 11 *July* 1960)

Abstract—Forty-three substituted phenyl phosphorus compounds were studied in relation to selective esterase inhibition in mice, ability to potentiate the toxicity of malathion, and metabolic conversion to more active antiesterase agents. The malathion potentiation and the degree of *in vivo* inhibition of the aliphatic esterases hydrolyzing malathion were generally related. A similarity appeared among compounds containing the *o*-methyl-, *o*-ethyl- and *p*-ethylphenyl groupings in their activity for producing neurotoxicity in hens (activity as recorded in the literature) and for potentiation of malathion in mice. However, several compounds lacking the *o*-methyl- and *o*-ethyphenyl groupings were quite selective aliphatic esterase inhibitors and were active in malathion potentiation in mice but did not yield a neurotoxicity with hens. The most active potentiator of malathion by the assay method used was di-*o*-tolyl mono-*p*-tolyl phosphate. Metabolic activation to more potent esterase inhibitors was demonstrated for many aryl phosphates containing alkyl substituents in several positions on the phenyl group.

TRI-ARYL PHOSPHATES are widely used as plasticizers, solvents, flame retardants, additives to extreme pressure lubricants and as lead scavengers in gasoline. The activity of tri-*o*-cresyl phosphate (TOCP) and certain other substituted phenyl phosphates in the production of a neurotoxicity characterized by peripheral and central demyelination in certain vertebrates is well established,[1–8] although the mechanism of this action is not clear.[4–6, 9–11] TOCP, presumably through the action of metabolites,[5, 12] is a highly selective *in vivo* inhibitor of certain aliphatic esterases and "pseudo-cholinesterases".[4, 9, 11, 13–17] Formation of active antiesterases from TOCP occurs *in vivo*,[5, 12] and can be effected by liver slices *in vitro*[5] and presumably by a rat liver soluble plus microsome system fortified with diphosphopyridine nucleotide and magnesium ions.[18] Another unusual biological activity displayed by TOCP[19, 20] and its thiono analog[20] is an ability to potentiate the toxicity to rodents of malathion[19, 20] and certain other carboxyester and amide-containing organophosphates.[20] This potentiation presumably depends on the selective inhibition by TOCP metabolites of esterases involved in the detoxification of the potentiated compounds.[19, 20] It has been suggested that agents capable of inhibiting hydrolytic detoxification might render people hypersensitive to the toxic effects of certain ester-type drugs.[21]

The specificity of tri-aryl phosphates and related compounds was further investigated with mice in relation to their conversion to more potent anti-esterase agents, *in vivo* and *in vitro* specificity in esterase inhibition, and ability to potentiate the toxicity of malathion. Since evidence is available that the organophosphate-induced ataxia in chickens may result from an esterase inhibition, the structure–activity relationship for this ataxia as reported in the literature is compared with that for esterase inhibition in mice.

METHODS AND MATERIALS

Compounds

The substituted phenyl phosphorus compounds studied and their sources are indicated in Table 1. All compounds were used as received without further purification and the results should therefore be comparable with other studies[6, 8, 22] where these same materials were used. Malathion (O:O-dimethyl-S-(1:2-dicarboethoxyethyl)-phosphorodithioate) was a sample of 96 per cent purity provided by the American Cyanamid Co., Stamford, Connecticut, U.S.A.

Treatment of mice

Twenty-five-gram female white mice from the Rolfsmeyer Farms (Madison, Wisconsin, U.S.A.) were treated intraperitoneally while under light ether anesthesia. Corn oil solutions of the phosphates were used, and in certain cases it was necessary to warm the corn oil to effect solution of the phosphorus compound. Injection volumes were 0·125 ml per 25-g mouse for the substituted phenyl phosphates and 25 μl per 25-g mouse for malathion.

A standard 100 mg/kg dosage of triaryl phosphate was administered 24 hr prior to either sacrifice of the mice for esterase activity assays or treatment with malathion for toxicity potentiation studies. Control mice were treated with corn oil only. None of the aryl phosphorus compounds produced any mortality or symptoms of poisoning within 3 days after injection of this 100 mg/kg dosage. Twenty or more mice were used in determining the toxicity of malathion administered 24 hr after each of the aryl phosphates. LD_{50} values as mg toxicant per kg mouse were estimated from logarithm–probit mortality plots. The LD_{50} 24 hr after administering malathion was about 1500 mg/kg with control mice and from 59 mg/kg to greater than 1200 mg/kg for mice pretreated with the aryl phosphorus compounds.

Enzyme determinations

Mouse tissues were removed rapidly following sacrifice. Heparinized blood was diluted 1:4 with bicarbonate buffer (0·0357 M $NaHCO_3$ and 0·164 M NaCl) and centrifuged to separate the corpuscles which were discarded. This 1:4 diluted plasma was used at 0·4 ml for assay with malathion as a substrate and at 0·1 ml with propionyl choline as a substrate. Homogenates of whole brain or liver were prepared at 20 per cent w/v in bicarbonate buffer with a Potter–Elvehjem type glass homogenizer and 0·4 ml homogenate was used for assay with each of the substrates.

Esterase activity was determined indirectly by CO_2 evolution from a bicarbonate buffer (0·0357 M $NaHCO_3$ and 0·164 M NaCl) in an atmosphere of 5 per cent CO_2 and 95 per cent N_2 at 38 °C in 10-ml Warburg flasks. The enzyme source in 0·40 ml was usually added to the side arm of the flask, and the substrate in 1·60 ml to the main compartment. The enzyme with or without added inhibitor was incubated for 20 min at 28 °C and 40 min at 38 °C before tipping to mix it with the substrate.

The esters at 0·0125 M were added in 1·60 ml to the main compartment of the flask so that the final concentration after tipping the enzyme was 0·01 M. Solutions of acetyl-β-methylcholine chloride, propionylcholine-*p*-toluene sulfonate (PrCh) and acetylthiamine chloride hydrochloride were prepared directly in the bicarbonate buffer. Emulsions of tributyrin and 96 per cent pure malathion and a suspension of testosterone propionate (test. pr.) were prepared by adding Triton X-100 (Rohm and

TABLE 1. STRUCTURE–ACTIVITY RELATIONSHIPS FOR CERTAIN ARYL PHOSPHORUS COMPOUNDS

Compounds*†	Ataxia in chickens‡ (1 g/kg oral)	Mouse LD_{50} malathion	% inhibition *in vivo* 24 hr post-treatment					Liver microsome activation§	
			Plasma malathion	Liver malathion	Plasma PrCh	Liver toc. ac.	Liver test. pr.	−DPNH	+DPNH
Symmetrical triaryl phosphates									
1. $(PhO)_3P(O)$	No[2, 6, 7]	450	62	50	31	0	0	57	52
2. $(2\text{-}MePhO)_3P(O)$	Yes[1, 2, 5–8]	190	97	85	94	97	73	+3	93
3. $(3\text{-}MePhO)_3P(O)$	No[2, 5–8]	340	92	59	92	17	22	80	88
4. $(4\text{-}MePhO)_3P(O)$	No[2, 5–8]	230	81	61	0	37	23	4	2
5. $(2\text{-}EtPhO)_3P(O)$	No[6, 8]	510	87	69	76	49	20	7	91
6. $(3\text{-}EtPhO)_3P(O)$	No[8]	140	86	76	83	0	0	77	84
7. $(4\text{-}EtPhO)_3P(O)$	Yes[8]	80	91	82	63	53	0	4	43
8. $(2\text{-}n\text{-}PrPhO)_3P(O)$	No[8]	>1200	35	48	0	0	0	3	21
9. $(2{:}3\text{-}Me_2PhO)_3P(O)$	No[22]	200	79	73	87	30	0	2	82
10. $(2{:}4\text{-}Me_2PhO)_3P(O)$	No[6]	1100	0	0	0	0	0	1	60
11. $(2{:}5\text{-}Me_2PhO)_3P(O)$	No[6, 7, 22]	1150	35	60	76	41	0	25	80
12. $(2{:}6\text{-}Me_2PhO)_3P(O)$	No[6, 7, 22]	1100	34	44	18	0	0	10	22
13. $(3{:}4\text{-}Me_2PhO)_3P(O)$	No[7, 22]	370	83	64	40	0	0	33	57
14. $(3{:}5\text{-}Me_2PhO)_3P(O)$	No[6, 7, 22]	760	85	51	30	0	0	41	50
15. $(2\text{-}Me\text{-}4\text{-}EtPhO)_3P(O)$	No[8]	850	83	53	16	0	0	3	2
16. $(2{:}4/2{:}5\text{-}Me_2PhO)P(O)$	No[22]	1100	70	75	70	44	25	8	78
Asymmetrical triaryl phosphates									
17. $(PhO)_2P(O)(OPhCl\text{-}2)$	No[6]	200	94	59	40	40	0	88	80
18. $(PhO)_2P(O)(OPhMe\text{-}2)$	Yes[6, 7]	180	46	69	85	83	57	26	91
19. $(2\text{-}MePhO)_2P(O)(OPhMe\text{-}4)$	Yes[6, 7]	54	95	82	92	97	82	6	82
20. $(4\text{-}MePhO)_2P(O)(OPhMe\text{-}2)$	Yes[6–8]	110	93	77	95	86	76	6	80
21. $(4\text{-}MePhO)_2P(O)(OPhEt\text{-}2)$	Yes[8]	80	95	88	93	87	75	8	80
22. $(4\text{-}MePhO)_2P(O)(OPhEt\text{-}4)$	No[8]	160	82	78	12	76	38	+8	12
23. $(2\text{-}EtPhO)_2P(O)(OPhMe\text{-}4)$	Yes[8]	170	91	73	79	70	30	+4	84
24. $(3\text{-}EtPhO)_2P(O)(OPhEt\text{-}4)$	No[8]	120	78	82	83	28	16	47	75
25. $(4\text{-}EtPhO)_2P(O)(OPhMe\text{-}4)$	No[8]	180	90	87	42	89	64	+1	32
26. $(4\text{-}EtPhO)_2P(O)(OPhPr\text{-}n\text{-}2)$	Yes[8]	120	94	85	92	79	49	6	49
27. $(2\text{-}n\text{-}PrPhO)_2P(O)(OPhMe\text{-}4)$	No[8]	440	89	53	0	0	0	+4	55
28. $(3{:}5\text{-}Me_2PhO)_2P(O)(OPh)$	No[22]	>1200	17	12	14	25	0	7	18
29. $(3{:}5\text{-}Me_2PhO)_2P(O)(OPhMe\text{-}2)$	Yes[7, 8]	320	89	87	72	0	0	3	77
30. $(3{:}5\text{-}Me_2PhO)_2P(O)(OPhEt\text{-}2)$	Yes[8]	>1200	30	0	0	0	0	12	9
31. $(3{:}5\text{-}Me_2PhO)_2P(O)(OPhEt\text{-}4)$	No[8]	280	92	45	45	0	0	35	36
32. $(3{:}5\text{-}Me_2PhO)_2P(O)(OPhPr\text{-}n\text{-}2)$	No[8]	320	90	67	71	0	0	24	80

TABLE 1.—*continued*

Compounds*†	Ataxia in chickens‡ (1 g/kg oral)	Mouse LD_{50} malathion	% inhibition *in vivo* 24 hr post-treatment					Liver microsome activation§	
			Plasma malathion	Liver malathion	Plasma PrCh	Liver toc. ac.	Liver test. pr.	−DPNH	+DPNH
Triaryl phosphorothionates									
33. $(2\text{-MePhO})_3P(S)$	Yes[2]	>1200	0	18	15	20	0	+6	13
34. $(4\text{-MePhO})_3P(S)$	—	>1200	0	0	0	0	0	1	2
Triaryl phosphites									
35. $(PhO)_3P$	—	100	92	75	63	78	76	88	88
36. $(4\text{-MePhO})_3P$	—	550	77	57	32	32	17	59	57
Dialkyl-*o*-tolyl phosphates									
37. $(EtO)_2P(O)(OPhMe\text{-}2)$	No[6]	>1200	53	0	65	45	0	+1	12
38. $(n\text{-PrO})_2P(O)(OPhMe\text{-}2)$	No[22]	>1200	0	20	55	29	0	+6	54
39. $(2\text{-Et-hexyl-O})_2P(O)(OPhMe\text{-}2)$	No[6]	1050	60	0	67	0	0	8	35
Others									
40. $(2\text{-MePhO})_2P(O)OP(O)(OPhMe\text{-}2)_2$	No[22]	840	52	0	63	0	0	27	45
41. $(3{:}5\text{-Me}_2PhO)_2P(O)Cl$	No[6]	660	88	43	83	53	0	27	33
42. Ph_3P	—	>1200	0	0	0	0	0	3	10
43. $Ph_3P(S)$	—	>1200	0	0	0	0	0	5	11

* Abbreviations: Me = methyl; Et = ethyl; Pr = propyl; Ph = phenyl; 2:4/2:5 for compound 16 designates a mixture of these xylenols used in synthesis.

† The compounds were obtained from the following sources: Compounds 3–9, 11–16, 20–32, 38 and 40 from Dr. H. F. Bondy, Coalite and Chemical Co., England; Compounds 1, 10, 18, 19, 37, 39 and 41 from Dr. R. Hansberry, Shell Development Co., Modesto, California, U.S.A.; Compounds 2, 17 and 33–36 from Eastman Organic Chemicals Dept., Eastman Kodak Co., Rochester, New York, U.S.A.; and compounds 42 and 43 from Dr. Helmut Tietz, Biological Laboratories, Farbenfabriken Bayer, Leverkusen-Bayerwerk, Germany.

‡ Superscripts refer to literature cited.

§ Aryl phosphorus compounds at 1×10^{-4} M were incubated with mouse liver microsomal fraction + DPNH, and the inhibitors formed were assayed with mouse plasma propionylcholinesterase. Results are presented as % cholinesterase inhibition after incubation with 1×10^{-5} M total aryl phosphate equivalent. The difference between the − DPNH and the + DPNH should be used in interpreting the degree of activation.

Haas Co., Philadelphia, Pennsylvania, U.S.A.) in acetone to dissolve the ester, evaporating the solvent and then adding the buffer with thorough mixing to yield a final concentration of 1·1 mg/ml Triton X-100. An emulsion of tocopherol acetate (toc. ac.) was prepared in the same manner except that 5·5 mg/ml Triton X-100 were used. For tissue blanks the substrate was replaced with buffer, and for substrate blanks the tissue was replaced with buffer. The high emulsifier level apparently caused no interference in the assays.

The b_{30} values (μl CO_2 liberated/30 min) under the assay conditions for the brain tissue were 113 with acetyl-β-methylcholine, 239 with tributyrin and 49 with acetyl thiamine; for the plasma were 50 with malathion and 75 with propionylcholine; and for the liver were 64 with malathion, 68 with tocopherol acetate and 53 with testosterone propionate.

For assay of the inhibitors *in vitro*, the aryl phosphates were added in acetone solutions to the side arm of the Warburg flask, the acetone evaporated and the enzyme preparation added. After mixing to aid solution of the inhibitor and enzyme in the side arm, the concentration of the aryl phosphate was from 1×10^{-4} to 1×10^{-7} M. All concentrations stated for inhibitors are those during the inhibition reaction and not during the assay of the esterase.

Metabolism to more potent esterase inhibitors

The ability of suitably fortified liver fractions to metabolize the aryl phosphorus compounds was investigated. Immediately after removing the liver from mice or chickens, 14 per cent (w/v) homogenates in cold 0·05 M potassium phosphate buffer at pH 7·0 were prepared in a chilled glass homogenizer. After preliminary centrifugation at 15,000 *g* for 30 min at 2 °C to remove the cell debris and mitochondria, the supernatant was recentrifuged for 60 min at 105,000 *g* and 2 °C. The supernatant from this 105,000 *g* centrifugation was designated the "soluble" and the precipitate the "microsome" fraction. The microsome fraction was reconstituted with similar phosphate buffer to the original homogenate volume. A single study was made using the chicken spinal cord with a similar fractionation procedure.

The aryl phosphorus compound in acetone was added to a 25 ml Erlenmeyer flask and the solvent evaporated to leave a residue of 0·2 μmoles aryl phosphate on the bottom of the flask. To this flask was then added 0·33 ml, 0·05 M, pH 7·0 potassium phosphate buffer, 0·67 ml water containing no cofactor or 3·6 μmoles di- or tri-phosphopyridine nucleotide (DPN or TPN) or their reduced forms (DPNH or TPNH), and 1·0 ml of the microsome, soluble, or microsome plus soluble preparation. Two hours incubation with shaking in air at 38 °C were then allowed for metabolism of the aryl phosphate. Two milliliters acetone were then added and the resulting precipitate removed by centrifugation. The acetone was removed under reduced pressure and aliquots of the remaining aqueous material added to the esterase source. The activity of esterase inhibitors formed by the liver fractions was assayed as described above. Suitable controls with mouse liver fractions showed no inhibition resulting with this procedure except that due to the organophosphate.

In a few studies the ease of extraction into *n*-hexane was determined for the anti-esterase products from TOCP. The products from *in vitro* activation, after precipitatating the proteins with acetone and removing the precipitate and the acetone, were assayed against mouse plasma propionylcholinesterase. The remaining supernatant

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was partitioned with an equal volume of *n*-hexane and the antiesterase agents recovered in each phase were similarly assayed. The hexane was removed at room temperature with a mild air jet prior to esterase assay. Products from the *in vivo* activation of TOCP were also studied 2 and 24 hr after injection of mice with 500 mg/kg TOCP in corn oil. The liver and small intestine were removed, homogenized in acetone, the acetone-soluble fraction recovered and after evaporation of the solvent, the residue partitioned between an equal volume of water and *n*-hexane. Aliquots of each phase were used for assaying the activity in inhibiting mouse plasma propionylcholinesterase.

RESULTS

In vivo *studies*

Many compounds were highly active in potentiating malathion (i.e. potentiated LD_{50} of less than 200 mg/kg) and in effecting ataxia in hens (compounds 2, 7, 18–21, 23 and 26 of Table 1). These compounds contained at least one 2-methyl, 2-ethyl or 4-ethyl substituent on a phenolic group. Only the 2-methylphenyl and 2-ethylphenyl di-(3:5-dimethylphenyl) phosphates (compounds 29 and 30) and the thiono analogue

TABLE 2. CORRELATION OF MALATHION TOXICITY AND INHIBITION OF MALATHION-HYDROLYZING ESTERASES 24 HR AFTER INJECTION OF MICE WITH ARYL PHOSPHORUS COMPOUNDS AT 100 MG/KG

LD_{50} malathion	No. of compounds	Ave. % inhibition malathion hydrolysis *in vivo*	
		Plasma	Liver
54–80	3	94	83
100–140	5	89	79
160–190	5	81	78
200–320	6	88	65
340–450	4	82	57
510–850	6	79	46
1050–1150	5	40	36
>1200	9	15	11

Summary of data presented in Table 1.

of TOCP (compound 33) produce ataxia in hens, but were not highly active in potentiating malathion toxicity to mice. These three compounds are less toxic to chickens than most of the other ataxia-producing materials.[2, 7, 8] Moderate to high activity in malathion potentiation (potentiated LD_{50} of less than 350 mg/kg) without activity for producing ataxia in hens resulted with ten compounds (3, 4, 6, 9, 17, 22, 24, 25, 31 and 32 of Table 1), none of which contained 2-methylphenyl or 2-ethylphenyl groupings with the exception of tri(2:3-dimethylphenyl) phosphate (compound 9).

The activity of the aryl phosphorus compounds in producing *in vivo* inhibition of the malathion-hydrolysing esterases of liver and plasma was generally related to their ability to increase the susceptibility of mice to malathion (Table 2). This correlation was better with the liver than with the more sensitive plasma esterase.

From the specificity of *in vivo* esterase inhibition by the aryl phosphates it is evident that malathion and propionyl choline are largely hydrolyzed by different enzymes of mouse plasma, and that malathion is hydrolyzed by a different liver enzyme than

tocopherol acetate or testosterone propionate, although the latter two substrates may be hydrolyzed at least partially by the same enzyme. The compound specificity for *in vivo* inhibition of the liver and plasma malathion-hydrolyzing enzymes was somewhat similar.

None of the substituted phenyl phosphorus compounds studied effected significant inhibition of the activity of brain homogenates in hydrolyzing acetyl-β-methylcholine or acetyl thiamine when the brains were removed for assay 24 hr after injection of a 100 mg/kg dose. The brain enzyme hydrolyzing tributyrin was more sensitive such that 50–65 per cent inhibition resulted with compounds 18–20, 30–40 per cent with 2 and 23, and 20–30 per cent inhibition with phosphates 6, 9, 21, 24, 25, 27 and 38, while no significant inhibition resulted with the other materials. The specificity for *in vivo* inhibition of the brain tributyrin-hydrolyzing esterase was different than that for inhibition of the plasma and liver enzymes studied. It must be noted that these analyses on *in vivo* inhibition of the brain esterases are based on single and not replicated analyses.

The approximate toxicity to mice was determined for the aryl phosphorus compounds that were most active in increasing the toxicity of malathion (i.e. potentiated LD_{50} of 200 mg/kg or less). LD_{50} values for these phosphates 72 hr after injection in corn oil solutions were mostly greater than 1000 mg/kg (compounds 2, 6, 9, 17, 19, 22 and 24–26). Compound 18 yielded an LD_{50} of 800–1000 mg/kg, compound 7 was 400–600, compounds 21, 23 and 35 were 300–400 and compound 20 was 200–300 mg/kg.

Preliminary studies showed that TOCP injected at 400 mg/kg increased the susceptibility of mice to several chemicals injected 24 hr later. The compounds affected and the approximate factor of decrease in their mg/kg LD_{50} are as follows: malathion, 10×; the insecticide, dimethoate (O:O-dimethyl-S-(N-methylcarbamoylmethyl)-phosphorodithioate), 5×; butyrylcholine, 6×; and 2-dimethylaminoethyl acetate, 3·5×. Less than a two factor change in susceptibility resulted with the following injected compounds: thiamine, acetylthiamine, atropine, veratrine, protoveratrine and the insecticides DDVP (O:O-dimethyl-2:2-dichlorovinyl phosphate) and Dowco 109 (O-4-*tert*-butyl-2-chlorophenyl-O-methyl methylphosphoroamidothioate).

In vitro *studies*

Studies on the sensitivity of certain of the esterases to *in vitro* inhibition by ten aryl phosphorus compounds are presented in Table 3. In no case was the liver esterase which hydrolyzes malathion greatly inhibited by 1×10^{-4} M aryl phosphate. The plasma enzymes hydrolyzing propionyl choline and particularly malathion were more sensitive. The selectivity for inhibiting the plasma malathion-hydrolyzing esterase compared with the cholinesterase (pI_{50} malathion-esterase/pI_{50} PrCh-esterase) was usually greater with the phosphates than with the phosphites studied.

Under suitable conditions, fractions from mouse liver homogenates were effective in converting TOCP to more efficient inhibitors of mouse plasma cholinesterase. The microsome fraction fortified with either DPNH or TPNH was active in this respect, as was the microsome plus soluble fraction fortified with DPN or TPN. The oxidized or reduced forms of the pyridine nucleotides were about equally effective as cofactors for the microsome plus soluble fraction, while the reduced forms were much more effective than the oxidized forms with the microsome fraction. No great

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difference was noted in the activity of the di- and tri-phosphopyridine nucleotides but the concentration of cofactor was not varied in these studies.

A comparison was made of the mouse plasma cholinesterase inhibitors formed from TOCP *in vitro* and those present in the liver and small intestine of mice 2 or 24 hr after injection of 500 mg/kg TOCP. The only criterion used in this comparison was the partitioning of the inhibitors between equal volumes of *n*-hexane and water. The inhibitors recovered from the liver *in vivo* partitioned greater than 90 per cent into an equal volume of hexane from water, the inhibitors from small intestine were less

TABLE 3. INHIBITORY ACTIVITY *in vitro* OF CERTAIN ARYL PHOSPHORUS COMPOUNDS ON MOUSE PLASMA CHOLINESTERASE AND THE MOUSE PLASMA AND LIVER ENZYMES HYDROLYZING MALATHION*

Compound	Mouse plasma-pI_{50}	
	PrCh	malathion
$(PhO)_3P(O)$	5·3	6·2
$(3\text{-}MePhO)_3P(O)$	5·7	6·1
$(4\text{-}MePhO)_3P(O)$	<4·0	5·4
$(3\text{-}EtPhO)_3P(O)$	5·0	6·3
$(4\text{-}EtPhO)_3P(O)$	<4·0	5·2
$(4\text{-}MePhO)_2P(O)(OPhEt\text{-}4)$	<4·0	5·4
$(4\text{-}EtPhO)_2P(O)(OPhMe\text{-}4)$	<4·0	5·2
$(PhO)_2P(O)(OPhCl\text{-}2)$	5·6	6·4
$(PhO)_3P$	5·4	6·0
$(4\text{-}MePhO)_3P$	4·7	4·5

* The pI_{50} (negative logarithm of molar concentration producing 50 per cent inhibition) for these compounds with the mouse liver enzyme hydrolyzing malathion was in every case greater than 4·0.

completely recovered in the hexane, and those formed by either fortified microsome plus soluble or the microsome fraction alone partitioned about equally between hexane and water. In studies on the nature of the *in vivo* metabolites of TOCP in rats it has been found[23] that at least three metabolites can be partially to completely extracted into hexane. It appears likely that the most active metabolite formed *in vivo* is the same as that formed by the microsome–DPNH preparation *in vitro* and that it is susceptible to further metabolism by hydrolysis and possibly oxidation.

Certain structural requirements are evident (Table 1) for the conversion of aryl phosphates to more potent mouse plasma propionylcholinesterase inhibitors (activation) by the mouse liver microsome-reduced pyridine nucleotide system. By comparison of the inhibitors present after incubation in the presence and absence of DPNH, the following relationships can be seen: (1) For symmetrical monosubstituted triaryl phosphates, the greatest degree of activation results with 2-methyl or 2-ethyl substituents although compounds with 2-*n*-propyl or 4-ethyl substituents are also activated. (2) For asymmetrical monosubstituted triaryl phosphates the same requirements hold for a 2-methyl or 2-ethyl group for maximum activation and 2-*n*-propyl or 4-ethyl group for activation. (3) This relationship did not hold for the di-(3:5-dimethylphenyl)-mono- (2 or 4-alkylphenyl)-phosphates since the 2-methyl and 2-*n*-propyl derivatives were activated but not the 2- or 4-ethyl phenyl derivatives. (4) With

symmetrical tri(dialkylphenyl)-phosphates the degree of activation varied greatly with the position of the substituents such that activation decreased in the order: 2:3 > 2:4 or 2:5 > 2:6; 3:4 or 3:5. The 2:4-dimethylphenyl phosphate and a mixed 2:4/2:5-dimethylphenyl phosphate were highly activated but not the 2-methyl-4-ethyl analog. (5) Other 2-methylphenyl phosphates activated were the O:O-dialkyl-*o*-cresyl phosphates and tetra-*o*-cresyl pyrophosphate. (6) No activation resulted with two triaryl phosphites, and with triphenyl phosphine and its sulfide, however, this does not rule out the possible activation of other phosphines or phosphites since the analogous phosphates for the derivatives studied were not activated. (7) The high inhibition resulting with tri-*m*-cresyl phosphate and tri-*m*-ethylphenyl phosphate in the absence of DPNH made it impossible to ascertain under these experimental conditions whether or not activation had occurred with these *m*-alkylphenyl phosphates.

The microsomal fraction from chicken liver was also effective in the activation of TOCP and tri-*p*-ethylphenyl phosphate (Table 4). With TOCP the active metabolite

TABLE 4. ACTIVATION OF TOCP AND TRI-*p*-ETHYLPHENYL PHOSPHATE BY CHICKEN LIVER MICROSOMAL FRACTION WITH OR WITHOUT DPNH FORTIFICATION

(Aryl phosphates at 1×10^{-4} M were incubated with microsomal fraction ±DPNH, and the inhibitors formed were assayed at 1×10^{-5} M with cholinesterase; results as b_{30} with 0·01 M PrCh at 38 °C are the average of duplicate runs, each of which showed the same effect of DPNH on the inhibitor formation.)

Esterase source	Esterase control	$(2\text{-MePhO})_2P(O)$		$(4\text{-EtPhO})_2P(O)$	
		−DPNH	+DPNH	−DPNH	+DPNH
Chicken spinal cord (20 mg/ml)	154	113	83	110	95
Chicken plasma (100 μl/ml)	185	147	49	142	145
Mouse plasma (25 μl/ml)	63	35	8	27	18

A similar study was made using a fraction from chicken spinal cord prepared and assayed in an identical manner to the liver microsomal fraction. No inhibition of any of the esterases resulted with any of the treatments.

could be detected with chicken spinal cord cholinesterase or with cholinesterase of mouse or chicken plasma. The metabolite of tri-*p*-ethylphenyl phosphate was apparently inactive when assayed with chicken plasma cholinesterase. No activation of either substrate occurred with a fraction from chicken spinal cord which was separated under the same centrifugation conditions used to obtain the liver microsome fraction.

DISCUSSION

Potentiation of the toxicity to mammals of certain organophosphates in mixtures presumably results from one compound interfering with the detoxification of a second compound. It has been shown by a variety of procedures[19–21, 24–28] that malathion is potentiated by those organophosphates which selectively inhibit an esterase

active in hydrolysis of one or both of the carboethoxy groups of the malathion molecule. This mechanism for malathion potentiation is generally supported by the evidence presented in the present study.

The structure–activity relationships for aryl phosphates in effecting potentiation of malathion toxicity to mice and in producing ataxia in hens are somewhat similar but certainly not identical. If both types of biological activity are related to esterase inhibition, then the effective antiesterases are present in different concentrations in the two organisms or the antiesterases differ in their selectivity for inhibiting the physiologically important esterases involved. There is considerable evidence[11] that true and pseudocholinesterase inhibition is not involved in the production of ataxia in hens. The failure of an ataxia-producing dose of tri-*p*-ethylphenyl phosphate to produce cholinesterase inhbition in hens has been used as evidence[10] for discarding the "pseudocholinesterase inhibition" hypothesis for ataxia production. However, a metabolite of tri-*p*-ethylphenyl phosphate formed *in vitro* by either the mouse or chicken liver microsomal fraction is an effective inhibitor of certain esterases, but apparently not for the chicken plasma cholinesterase. It is also known that certain compounds inactive in producing neurotoxicity in hens are active in effecting paralysis in cats.[2]

Numerous investigators have been intrigued with the great selectivity in esterase inhibition in animals treated with various organophosphorus esters. Many phosphate esters with ammonium or sulfonium groupings are highly selective for cholinesterase inhibition, and others such as certain bis-aryl phosphoramidates are selective for aliphatic esterases.[29, 30] In the present study it was found that a high selectivity for aliphatic esterases resulted with triaryl phosphates containing only *p*-alkyl substituents such as methyl or ethyl.

Many esterases are inhibited by organophosphates. The normal physiological function of many of these enzymes is unknown. Just as the organophosphate cholinesterase inhibitors have proven invaluable in ascertaining the significance of the various reactions mediated by cholinesterase, so selective inhibitors of other carboxylic esterases should help ascertain the role of these esterases in the body. Certain aliphatic esterases are involved in drug metabolism and the potentiation between organophosphates has given a dramatic demonstration of this role. The significance of inhibition of esterases other than acetylcholinesterase in the acute and chronic toxicity of the varied organophosphates, and in the infrequently observed ataxia or neurotoxicity, and anesthetic activity[31] to mammals is not clearly established. Further studies are also needed on the inhibition of these other esterases in relation to the poisoning of insects by organophosphates and the acquired resistance of insects to these compounds, the poisoning of nematodes and other lower animals and the herbicidal action of phosphate esters, and the activity of some compounds of this type as anticarcinogenic agents.

TOCP and many other aryl phosphates are metabolized to more potent esterase inhibitors. The chemical nature of the active metabolites has not been determined. The reaction is probably at least initiated by an oxidation since it can be effected with a liver microsome-reduced pyridine nucleotide system in an oxygen-containing atmosphere. The active products can be readily demonstrated in rats, mice, chickens and cockroaches. The metabolite formed from TOCP *in vivo* by rats has now been

highly purified, and is active at 1×10^{-8} M as an inhibitor of human plasma cholinesterase.[23]

Acknowledgements—Approved for publication by the Director of the Wisconsin Agricultural Experiment Station. This investigation was supported by a grant from the National Institutes of Health, U.S. Public Health Service (Contract no. RG-5304). The skilled technical assistance of Miss Judith Engel, Mr. Kheong Wong and Mr. Donald Hintz and the helpful suggestions of Dr. Ernest Hodgson are gratefully acknowledged. Especial indebtedness is due to Dr. J. M. Barnes for his advice and encouragement in conducting these studies and for making the arrangements for procuring the majority of the compounds utilized.

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Biochemical Pharmacology, 1961, Vol. 5, pp. 343–344. Pergamon Press Ltd., Printed in Great Britain.

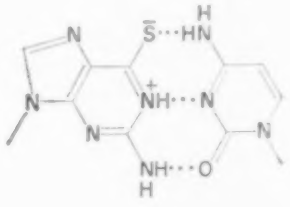
SHORT COMMUNICATIONS

6-Selenoguanine (2-amino-6-selenopurine). Synthesis and biological studies

(*Received* 5 *October* 1960)

6-THIOGUANINE has been studied extensively as an inhibitor of the growth of transplantable neoplasms in animals and to some extent as an antileukemic agent in man. It exhibits antimitotic activity[1] and has been shown to be incorporated into deoxyribonucleic acid (DNA).[2] While the mechanism of action of this compound has not as yet been established, it seems possible that charge separation of the type:

which has been found to be greater in thiocarbamyl than in carbamyl compounds,[3, 4] might lead to unusually strong hydrogen-bonding with the amino group of cytosine facing thioguanine in the double helix of DNA:



Such intra-helical interaction would be expected to interfere with the replication of deoxyribonucleic acid.

Since replacement of sulfur by selenium leads to an even more marked polarization in this type of structure and because 6-seleno-purine had shown some antitumor activity in mice,[5, 6] in spite of its lack of stability, the synthesis of 6-selenoguanine was of interest.

2-Amino-6-selenopurine was prepared by the reaction of sodium hydroselenide with 2-amino-6-chloropurine (generously supplied by G. B. Elion and G. H. Hitchings of the Wellcome Research Laboratories) in a manner analogous to that used in the synthesis of 6-selenopurine.[4]

Stability studies with 6-selenoguanine in buffered solutions showed that this compound is more stable than is 6-selenopurine. Effective inhibition of the growth of *Lactobacillus casei* (ATCC 7469) occurred with selenoguanine with one-tenth the concentration required with thioguanine.

While the single-dose toxicity of selenoguanine in mice exceeded that of its sulfur analog, the opposite proved to be the case when the compounds were administered repeatedly.

The effects of selenoguanine and thioguanine on the growth *in vivo* of several experimental murine neoplasms: lymphomas L1210 (sublines A and B) and L5178Y, and Sarcoma 180, were determined, and it was found that the antitumor activities of these two compounds were comparable. However, the data obtained thus far indicate that selenoguanine possesses an appreciably higher therapeutic index than does thioguanine. The response of mice bearing either lymphoma L1210 (subline A), lymphoma L5178Y, or Sarcoma 180 to seven or eight daily intraperitoneal injections of selenoguanine or thioguanine revealed that, during the period of treatment, the therapeutic indices of the two compounds were the same; i.e., the maximally tolerated dose of selenoguanine was about four times that of thioguanine, and the dose of selenoguanine required to attain a given degree of antitumor activity was also four times that of thioguanine. However, when these mice were observed during the week

following the final injections of the two compounds, it was found that significantly fewer animals treated with selenoguanine succumbed to what appeared to be delayed drug toxicity than was the case with mice treated with thioguanine. In mice bearing lymphoma L1210 (subline B) (kindly provided by Dr. J. H. Burchenal of the Sloan-Kettering Institute), the higher therapeutic index of selenoguanine, compared with that of thioguanine, was seen even during the week of treatment: the maximally tolerated dose of selenoguanine was eight times that of thioguanine, while the dose of selenoguanine required to attain a given level of antitumor activity was only twice that of thioguanine.

TABLE 1. SINGLE- AND REPEATED-DOSE TOXICITY OF SELENOGUANINE AND THIOGUANINE IN HA/ICR SWISS MICE

Compound*	Intraperitoneal dose (mg/kg)	Dose schedule	Average† change in body weight (g)	Mortality (1st week)	Mortality (2nd week)
Selenoguanine	100	single injection	−2·0	3/4	—
Thioguanine	120	single injection	−2·0	0/4	1/4
Selenoguanine	16	daily × 7	−2·0	0/10	0/10
Thioguanine	4	daily × 7	−3·5	0/10	8/10

* The compounds were suspended in a 0·25% solution of carboxymethylcellulose; the volume injected was constant, 0·4 ml.

† Mice were individually weighed and numbered at the onset of the experiment. The average initial weight of these animals was 20 g. On the day following the last injection (8th day), each mouse was reweighed, and the average change in weight of each group of 10 was recorded. On the average, mice of this age, strain, and sex (female), receiving daily intraperitoneal injections of 0·4 ml of a 0·25% solution of carboxymethylcellulose, gain 1–2 g.

It was also found that the unresponsiveness of a 6-mercapto-purine-resistant tumor extended to selenoguanine as well as to thioguanine.

All findings considered, selenoguanine appears to offer considerable promise as an inhibitor of tumor growth, and additional studies of its properties are in progress. This work was supported in part by grants (CY-3937) and (CY-2817(C4)) from the United States Public Health Service.

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SECOND SCANDINAVIAN SUMMER MEETING OF BIOCHEMISTRY, PHARMACOLOGY AND PHYSIOLOGY

August 27–29, 1959, Turku, Finland

AT THIS meeting papers were read on the following topics: the Biochemistry of Subcellular Particles; the Biochemistry of Connective Tissues; the Mechanisms of Glucuronide and Sulphate Conjugation; and the Central Control of the Autonomous Nervous System.

On the following pages are published papers selected from this Symposium; while a further and final selection will appear in Volume 6 Number 1 of Biochemical Pharmacology.

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Biochemical Pharmacology, 1961, Vol. 5, pp. 347–350. Pergamon Press Ltd., Printed in Great Britain.

ROLE OF STRUCTURE IN REGULATION OF CELLULAR ENZYMIC ACTIVITIES

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Abstract—Advances in enzymology have shown that a cell is not a mixture of enzymes, but a highly organized community where mitochondria, nucleus and endoplasmic reticulum are concerned in cellular activity. Quantities of enzymes appear to be of less importance in cell activity than correlations of enzymes with other factors such as structural barriers. Pyridinenucleotide-linked oxidations which take place in the extra-mitochondrial space of the cell proceed by way of extra-mitochondrial pyridinenucleotides. Experiments demonstrated that externally generated DPNH can pass the mitochondrial barrier to enter the respiratory chain. Fusion of enzymes to greater structural units would appear to give to the cell a level of organization between that of a single enzyme and the cellular entity.

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TO THE modern cytologist the mitochondrion is the classical concept in subcellular organization. The electron microscopical and enzyme chemical complement of this organelle has been known in its main details for almost 10 years. Its ability to produce phosphate bond energy in the isolated state is well known to every biochemist.

The importance of mitochondrial research is, however, not restricted to a more or less advanced knowledge of the biochemistry and physiology of that particular subcellular component. It has also, for the first time, given the biologist a way of expressing himself in defined chemical terms and thus make himself understood by scientists working in other fields. For the biochemist, the realization that a cell is not a random mixture of enzymes but a highly organized community where components such as mitochondria, nucleus and endoplasmic reticulum all have their role in the maintenance of cellular activity, has brought a new concept to enzymology.

With the statement that a cell is able to do what its enzymic complement allows, as background, great efforts have been made during the post-war period to correlate the amount of enzymes in a cell with its physiological condition. For the cancer cell this has been particularly relevant, and a bulk of analytical data on the content of enzymes in tumours compared to normal cells have been collected. Also the activity of many enzymes in correlation to hormonal status has been a subject of intense study. In spite of the fact that many significant differences have been obtained, the enthusiasm for this type of research has gradually decreased and been replaced by the feeling that differences in the metabolic pattern of a cell must be correlated to factors other than quantities of enzymes.

Some examples of this phenomenon will be given below.

Perhaps the most serious problem a cell has to face is how to master the excess of enzymic activities it has at its disposal. How, for example, can it avoid combusting all substrate with the very high oxidizing power which can be attributed to a cell, and thus leave some of it as building blocks for new cellular material? Why, for example is a

tumour but not liver able to accumulate lactic acid? The cells from both tissues have, as we know, a considerable amount of lactic dehydrogenase at their disposal. For the solution of these problems the existence of structural barriers in the cell have to be considered.

As is well known, isolated mitochondria can oxidize all members of the citric acid cycle without the addition of any cofactors. Lactate, on the other hand, is not able to give rise to any respiration even if lactic dehydrogenase is added to the medium. Only after the addition of DPN can the mitochondria combust lactate. The dinucleotides present in the mitochondria cannot function as cofactors in the external lactic dehydrogenase system. This phenomenon, which is illustrated in Table 1 by an experiment

TABLE 1. REQUIREMENTS FOR THE OXIDATION OF LACTATE BY HEART-MUSCLE MITOCHONDRIA
(AFTER VON KORFF AND TWEDT[1])

Additions	μlO_2
None	2·4
Pyruvate	49·3
Lactate	4·0
Lactate + LDH*	8·1
Lactate + DPN†	13·5
Lactate + LDH + DPN	55·4

* LDH = lacticdehydrogenase.
† DPN = diphosphopyridinenucleotide.

quoted from Von Korff and Twedt[1], is also true of other extramitochondrial dehydrogenases. There is, therefore, reason to believe that those pyridinenucleotide-linked oxidations which take place in the extramitochondrial space of the cell, among them the oxidation involved in glycolysis, proceed also in the living cell by way of extramitochondrial pyridinenucleotides.

The acceptance of this statement immediately leads to a new problem: How are the extramitochondrially generated reduced pyridinenucleotides, e.g. the glycolytic DPNH, further oxidized by oxygen? The importance of this is obvious if we consider that in an aerobic cell, for each molecule of pyruvate formed from glucose, one molecule of extramitochondrial DPNH must be reoxidized by oxygen, provided that no accumulation of lactate takes place and no reductive synthesis of DPNH occurs parallel to the glycolysis. For a long time the main way known by which extramitochondrial DPNH could be oxidized aerobically by mitochondria has been that first described by Lehninger[2], which requires the addition of extramitochondrial cytochrome *c*.

An experiment by Von Korff and Twedt[1] demonstrates how hydrogen from non-mitochondrial substrates can be oxidized by way of the mitochondrial respiratory chain. This is, however, hardly a physiological mechanism, since cytochrome *c* is not known to occur in the ground cytoplasm of the cell.

Perhaps a more physiological experiment to demonstrate how externally generated DPNH can pass the mitochondrial barrier to enter the respiratory chain is to use a mitochondrial substrate, the oxidized form of which can be re-reduced by DPNH

formed in the ground cytoplasm. Zebe *et al.*[3] give an elegant example of this (Fig. 1). α-Glycerolphosphate is a mitochondrial substrate from the flight muscles of insects. This substrate is oxidized by means of a mitochondrial flavoenzyme to dihydroxyacetonephosphate which by means of an external enzyme and DPNH is re-reduced to glycerolphosphate. In this way glycerolphosphate becomes a cofactor for the trans-

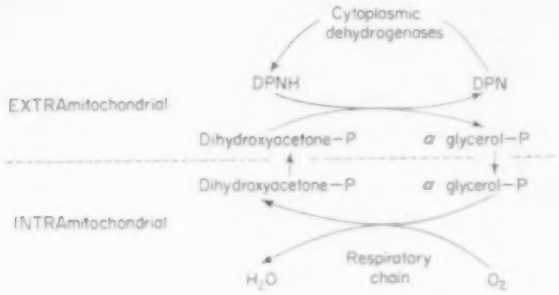

FIG. 1. Transport of hydrogen through mitochondrial barrier. (After Zebe, Delbrück and Bücher, *Biochem. Z.*, 1959).

portation of DPNH, formed in the ground cytoplasm, into the combustion oven of the mitochondrion.

The structure of this barrier between ground cytoplasm and mitochondria is a matter for discussion, but it seems certainly not to be a simple permeability problem.

An example of how structures can influence the activity of enzymes might be elucidated by the following experiment. To trap and conserve the energy formed

TABLE 2. ADSORPTION OF HEXOKINASE ON MITOCHONDRIA (AFTER SIEKEVITZ[5])

	Relative units
Added	100
Recovered in suspension after recentrifugation	89
Expected in pellet after recentrifugation	11
Found in pellet after recentrifugation	103

during mitochondrial oxidative phosphorylation, the addition of glucose and hexokinase is the best tool to use. This addition converts the ATP-formed into glucose-6-phosphate and ADP. In an attempt to calculate the amount of ATP present in the incubation medium from the differences in speed of formation and degradation, we obtained a much lower value for ATP than was expected.[4]

Siekevitz[5] working on the same system found that if the hexokinase is removed from the mitochondria by centrifugation, the minute amount of hexokinase which was adsorbed on the mitochondria, could after resuspension of the mitochondria in a fresh medium exert as high an activity as that originally added to the suspension. A considerable activation of hexokinase had evidently taken place. To explain this phenomenon is has been suggested that the hexokinase exerts its effect on the surface

of the mitochondrion. In this case a local high concentration of ATP might be responsible for the high enzymic activity. On the other hand, we know that only low concentrations of ATP are required for a high hexokinase activity in homogenized tissues. A further possibility is to apply the way of thinking introduced by McLaren and Bobcock[6]. These authors point out how surface structures can locally change the pH of a solution up to a whole unit, a phenomenon specially valid for cell surfaces. As hexokinase has its maximum activity at pH 8·5, and mitochondrial suspensions work at about pH 7·5, this offers a theoretical possibility for explaining the increased activity of the enzyme.

However these experiments are interpreted, they clearly demonstrate that the fusion of enzymes to greater structural units gives to the cell a level of organization between that of the single enzyme and the cellular entity. If this level of organization is going to be the background of hormonal control, physiological variation and cellular individuality will be a future subject of research.

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FRACTIONATION OF PARTICLES AND MACROMOLECULES IN AQUEOUS TWO-PHASE SYSTEMS

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Abstract—The isolation from cells of particles such as nuclei, mitochondria, microsomes and chloroplasts is important in biochemical and physiological studies. Cell particles are separated according to size, density and form. Experiments showed that surface properties of particles could determine the fractionation in a liquid-liquid two phase system, complementing conventional fractionation techniques. Aqueous polymer two phase systems were used in preference to organic solvent-water systems. Enzymes and viruses may be distributed in these systems without loss of activity. The usefulness of the method is demonstrated with examples. Other properties of polymer two phase systems are also discussed.

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THE study of mass isolated cell particles, e.g. nuclei, mitochondria, chloroplasts, microsomes and cell walls, obtained from disintegrated cells has been of considerable importance in recent developments in biochemistry and cell physiology. Such work requires efficient fractionation methods. In general, centrifugation techniques have been used, by which methods the particles are separated according to their size, density and form. It would, however, be of value if the centrifugation methods could be complemented by other methods whereby other properties of the particles, for example, the surface properties, determine the fractionation.

An example of such a method is the partitioning of particles in a liquid–liquid two-phase system. If a number of particles are shaken in a two-phase system they will distribute themselves largely according to their surface properties and collect either in the one or the other phase, or at the interface. The present review summarizes the work[1–11] that has been carried out at the Biochemical Institute of Uppsala for the purpose of developing methods for the fractionation of particles and macromolecules according to this principle.

THE PHASE SYSTEMS

Special problems arise when one is selecting phase systems for biochemical substances. The phase systems should be as inert as possible to the particles which are to be fractionated. This means that consideration must be paid to the water content, ionic strength, osmotic pressure, ability to dissolve out substances from the particles, denaturing effects on proteins, and so forth. Since adsorption of particles at the interface is sometimes used for fractionation, the possible damaging effect of the interfacial tension must also be considered. Generally, a low interfacial tension is desirable. All these different requirements usually rule out the common two-phase systems which contain an organic solvent such as the systems water–benzene, water–ether or water–butanol. Instead, a number of aqueous polymer two-phase systems have been studied.

These are composed of water and two water-soluble polymers. Solutions of two different polymers are usually not miscible even at low concentration of the polymers.[12] Thus for example if 1% (w/w) aqueous solution of dextran (a polyglucose) is mixed in a test tube with an equal amount of a 1% aqueous solution of methyl cellulose, one will observe that the mixture becomes turbid. If the tube is allowed to stand for a while, two liquid layers are formed. An analysis of these will show that the bottom layer contains most of the dextran and the top layer most of the methyl cellulose. If the mixture is shaken again and allowed to stand, the two layers will again separate out. One has thus obtained a system containing two immiscible phases in equilibrium, both consisting largely of water. The difference in the physical properties of the two phases is small, compared with ordinary phase systems. Thus the difference in density or refractive index of the two phases is very small, a fact which sometimes makes it difficult to detect the interface. The interface has a low surface tension as shown by the fact that the interface forms a right angle with the wall of the test tube.

A number of pairs of polymers which give a liquid two phase system when mixed with water are recorded in Table 1. The composition of a phase system and its phases

TABLE 1. PAIRS OF POLYMERS WHICH GIVE A LIQUID TWO-PHASE SYSTEM WHEN MIXED WITH WATER (FROM ALBERTSSON[2])

Polypropylene glycol–polyethylene glycol
Polyethylene glycol–dextran
Polyvinyl alcohol–dextran
Polyvinylpyrrolidone–dextran
Methyl cellulose–dextran

at different proportions of the polymers may be represented by a phase diagram. Figs. 1 and 2 show phase diagrams of the two systems which have been used for the fractionation of particles, namely the dextran–methyl cellulose–water and dextran–polyethylene glycol–water systems. Mixtures represented by points above the curve of

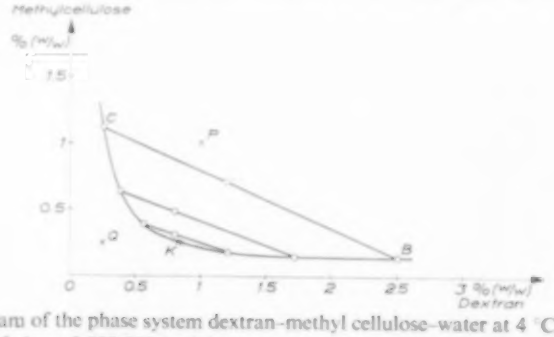

FIG. 1. Phase diagram of the phase system dextran–methyl cellulose–water at 4 °C. The dextran has a molecular weight of about 2,000,000 (weight average) and the methyl cellulose a molecular weight of about 150,000.

Fig. 1 result in two-phase systems while mixtures represented by curves below the curve result in a one-phase system. Thus a mixture of 1% (w/w) dextran and 1% (w/w) methyl cellulose (point *P* of Fig. 1) is a two-phase system while a mixture of 0·2% (w/w) dextran and 0·2% (w/w) of methyl cellulose (point *Q* of Fig. 1) gives an homogeneous solution. If a mixture represented by point *A* of Fig. 1 is set up and its phases

analysed, they will have the compositions represented by the points *B* and *C* (Fig. 1). Thus the points *A*, *B* and *C* represent the total composition, the composition of the bottom phase, and the composition of the top phase, respectively, of a given phase system. The points *A*, *B* and *C* lie on a straight line, the tie line. Figs. 1 and 2 show a number of such tie lines, connecting points which represent the composition of two

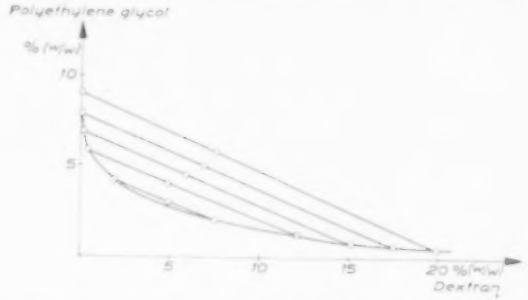

FIG. 2. Phase diagram of the phase system dextran–polyethylyne glycol–water at 0 °C. The dextran has a molecular weight of about 480,000 (weight average) and the polyethylene glycol a molecular weight of 6000–7500.

FIG. 3. Partition in a two-phase system

phases in equilibrium. The length of a tie line is a measure of the difference in composition of the phases. As may be seen in Fig. 1 and Fig. 2 as the composition approaches the point *K* the length of the tie lines becomes smaller and smaller. At the point *K* the compositions of the two phases become theoretically equal. The point *K* is called the critical point. At the critical point the property of the system is rather sensitive to changes in the composition of the phase components or the temperature. Therefore best results are usually obtained by the use of systems which have compositions removed from the critical point.

These aqueous polymer systems may be complemented by the addition of substances such as salts or sucrose which are necessary for the stability of the particles to be fractionated. They may be used in the cold as well as at room temperature.

It has been found that a number of enzymes may be distributed in these systems without any loss in activity. The enzymes so far tested include enolase, laccase, coeruloplasmin, cholinesterase and cytochrome oxidase.[3] Different viruses including the bacteriophages T_3 and T_2,[5] vaccinia virus, parotitis virus, Newcastle disease virus, influenza virus, echo virus, and adenovirus[9, 10] have also been tested. No virus inactivation was found to occur. Therefore, it appears that the systems are not harmful. This is probably, to a great extent, due to the fact that they have a high water content and a low interfacial tension. However, the phase components themselves

may also have a stabilizing action on the particles and enzymes. Dextran, methyl cellulose, and polyethylene glycol are all poly-ols, and it is a common experience that poly-ols such as sucrose and polysaccharides can stabilize proteins against denaturation. In fact, it has been found that the bacteriophage T_2 may be treated by a flourocarbon without loss if dextran is present in the phage suspension, while almost all the activity disappears if the polysaccharide is not present.[8]

THE BEHAVIOUR OF SOME PARTICLES AND PROTEINS

If a low molecular substance is partitioned in a two-phase system (Fig. 3) a finite partition coefficient, K, is usually established (K is the concentration of the partitioned substance in the top phase divided by the concentration of the substance in the bottom phase). It was shown by Brønsted[13] that the more the molecular weight of the partitioned substance increases, the more unilateral the distribution tends to be. Thus, for example, if a protein is distributed evenly between the two phases a number of identical cellular particles usually collects in only one of the phases. This is illustrated by Table 2 in which the partition coefficient for a number of proteins and virus

TABLE 2. PARTITION OF PROTEINS AND VIRUS PARTICLES IN A DEXTRAN–METHYL CELLULOSE SYSTEM (DATA FROM ALBERTSSON AND FRICK[5])

Protein or virus particle	Surface area of the particles $\times 10^{-3}$ $(m\mu)^2$	Partition coefficient
Haemocyanin "eighth"	0·86	0·63
Haemocyanin whole	3·5	0·25
T_3-bacteriophage	8·7	0·023
Tobacco mosaic virus	14·4	0·01–0·02
T_2-bacteriophage	25·5	0·0005
Vaccinia virus	220	0·0001

particles are recorded. It is evident that the larger particles like the bacteriophages and vaccinia virus have a concentration in the bottom phase 100–1000 times greater than in the top phase. Small particles like the haemocyanin and phycoerythrin molecules are more evenly distributed. A proportionality between the logarithm of the partition coefficient and the surface area of the particles has been found.[5] Fig. 4 shows how the

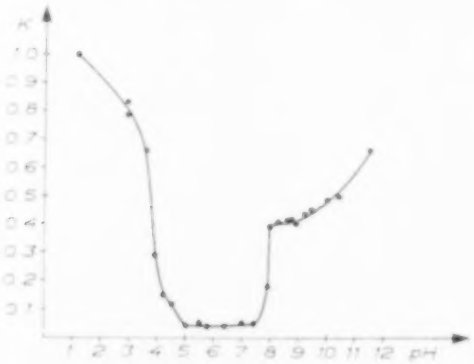

FIG. 4. The partition coefficient, K, of *Helix pomatia* haemocyanin in a dextran–methyl cellulose system as a function of pH.

partition coefficient of *Helix pomatia* haemocyanin depends upon the pH in the dextran–methyl cellulose system. The molecular weight of haemocyanin at different pH values is well known from studies in the ultracentrifuge.[14] Thus for pH 4·7–7·0 this protein has a molecular weight of $8{\cdot}9 \times 10^6$, and in this pH interval the *K*-value is constant (Fig. 4). Between 7·4 and 8·2 the molecules split partly into halves and eighths. For pH 8·2–9·3 the protein is again monodisperse with a molecular weight of 1×10^6, and in this interval the *K*-value is also almost constant. Above pH 9·3 the protein molecules split into smaller units. For pH 3·6–4·6 the protein molecules are partly dissociated into halves, and at pH 3·6 it is dissociated into still smaller units. Fig. 4 shows that these changes in the molecular state are well reflected in the partition coefficient. In a similar way the association between the antigen and the antibody in an immunological reaction may be followed in a two-phase system.[11]

All experiments which have been made with the dextran–methyl cellulose system indicate that the size, or more probably the surface area of the particles, is a dominating factor in determining the partition coefficient of proteins and viruses.

In the dextran–polyethylene glycol system the behaviour is very different. The distribution of particles and proteins depends very much upon the salt environment. Thus a substance may be transferred entirely from one phase to the other simply by replacing one ion with another. Phycoerythrin, for instance, is mainly in the top phase in the presence of phosphate ions. If these are replaced by chloride ions the phycoerythrin is transferred to the bottom phase.[7] This salt effect is specific so that one protein may be separated from another by chosing a suitable salt concentration.

Another characteristic of the dextran–polyethylene glycol is that larger particles, like virus particles and cells, are easily adsorbed at the interface. This adsorption at the interface of the particles is, like the partition coefficient of proteins, dependent on the salts present, and a particle may accordingly be transferred from one phase to the interface by changing the salt.

APPLICATIONS

Fractionation of rat brain microsomes

Microsomes consists essentially of two submicroscopic structures, vesicles formed by fragments of the cyloplasmic membranes, and small electron-dense particles. The isolation of the latter particles from a microsome suspension prepared from rat brain has been accomplished by using a dextran–methyl cellulose system.[4] Fig. 5 is a scheme for the fractionation procedure. The microsome suspension was first shaken in a dextran–methyl cellulose system of the same composition as that in Table 2. In that system both kinds of structures are partitioned in favour of the dextran-rich bottom phase ($K < 1$). The vesicles have, however, a very low partition coefficient so that they are almost entirely in the bottom phase. The particles have a larger partition coefficient, and because of the large volume of the upper phase a considerable fraction of them is present in this phase. Thus the upper phase contains only the particles. This phase is removed and replaced by fresh top phase and the shaking is repeated in order to extract more particles from the bottom phase. To speed up the phase separation the tubes are centrifuged. The two phases are then combined and transferred to a new centrifuge tube (II). Dextran and methyl cellulose are then added to this tube in order to produce a very small bottom phase into which the particles are concentrated. The

bottom phase is then collected by making a small hole in the bottom of the plastic centrifuge tube. The particles are then centrifuged down at a high speed.

The starting microsome material consisted of the whole supernatant from rat brain tissue which had been homogenized with 4 vols. of buffered sucrose (0·25 M sucrose, 0·001 M My, Cl_2 and K_2HPO_4) and centrifuged at 18,000 *g* for 15 min. This contained both the microsomal structures and the soluble cytoplasmic fraction. The latter was not removed until the final centrifugation step. Thus the microsomes had not at any stage been packed in the centrifuge at high speed, a procedure which was found to cause considerable irreversible aggregation.

Examination in the electron microscope, and chemical analysis of the protein and the RNA content of the starting and final preparations, showed that a considerable purification of the RNA-rich particles has been achieved (for details see Albertsson *et al.*[4])

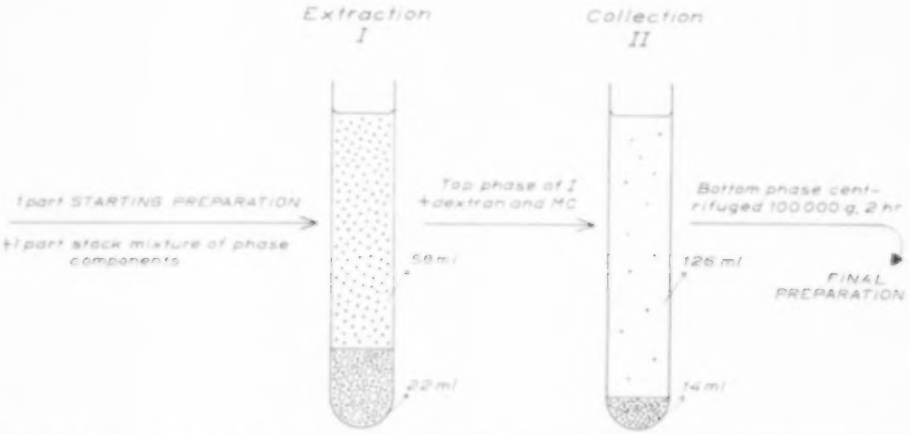

FIG. 5. Fractionation of rat brain microsomes in a two-phase system. Particles and vesicles are symbolized. (From Albertsson *et al.*[4].)

The concentration and purification of virus

Modern tissue culture techniques allow a production of virus suspensions on a 10 l. scale. Although these cultures are biologically highly active they are extremely dilute in terms of milligrams of virus material per millilitre. To concentrate the virus from these large volumes of diluted virus into a small volume is therefore an important step in the purification of a virus. Such a concentration can be accomplished in a two-phase system, provided that the virus particles give a fully one-sided distribution, and that the volume into which they are collected can be made small compared with the volume of the original culture suspension. A number of experiments with bacteriophages[5, 8] and animal viruses[9, 10] have shown that it is possible to construct a polymer phase system which fulfils these requirements. Table 3 shows the distribution of some viruses in such phase systems, which shows that the viruses are concentrated efficiently into the small bottom phase. The concentration is accomplished simply by the addition of suitable amounts of concentrated solutions of the polymers to the culture fluid. The mixture is then shaken and allowed to stand for phase separation. Concentration may then also be carried out in many steps so that a concentration of virus from, for example, from 10 l. to 10 ml can be achieved.

This technique has many advantages over centrifugation. First, packing of the virus particles together with impurities can be avoided. This occurs during high-speed centrifugation and it often leads to losses in virus activity. Second, since other substances such as proteins and cell fragments distribute in a way different to the virus

TABLE 3. CONCENTRATION OF SOME VIRUSES INTO THE SMALL BOTTOM PHASE OF A DEXTRAN-POLYETHYLENE GLYCOL SYSTEM (DATA FROM WESSLÉN *et al.*[9])

Virus	Titre per ml of bottom phase volume = 0·6 ml	Titre per ml of top phase volume = 9 ml	Virus assay
Vaccinia	7·6	4·9	Infectivity titres (log values)
Echo 7	9·5	5·5	Infectivity titres (log values)
Newcastle disease	2048	4	Hemagglutination titres
Influenza	4096	32	Hemagglutination titres
Parotitis	2048	4	Hemagglutination titres

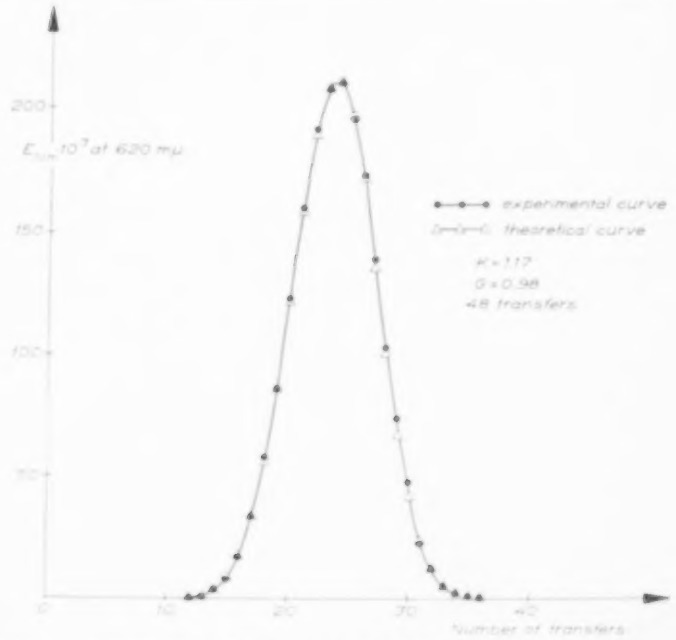

FIG. 6. Countercurrent distribution of phycoerythrin in a dextran–polyethylene glycol system. (From Albertsson and Nyns[6].)

particles, a considerable purification may be obtained by the phase system at the same time as the concentration is being accomplished. Third, the technique is very simple and can be applied on a large scale without the use of a complicated apparatus.

Counter-current distribution of proteins

The method of counter-current distribution has been shown to be one of the most efficient tools for analyzing and separating mixtures of biochemical interest. So far it

has mainly been applied to low molecular weight substances such as polypeptides but recently a number of smaller proteins have also been successfully distributed.[15] One of the advantageous features of counter-current distribution is that it is based upon several steps, each involving a state of equilibrium. Generally a state of equilibrium is supposed to be more easily obtained between two liquid phases than between, for example, a solid phase and a liquid phase. For macromolecules this difference in equilibrium behaviour may be expected to be more pronounced than for low molecular weight substances. It should therefore be of the greatest interest if counter-current distribution can be applied also for large molecular weight substances, such as proteins and nucelic acids.

Fig. 6 shows a counter-current distribution of a protein, phycoerythrin having a molecular weight of 300,000 in a dextran–polyethylene glycol system. The experimental curve fits the theoretical curve very well, indicating that the protein behaves

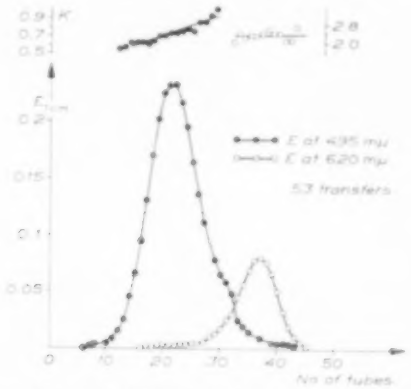

FIG. 7. Separation of phycoerythrin and phycocyanin in a dextran–polyethylene glycol system. (From Albertsson and Nyns[6].)

according to the Nernst distribution law. Fig. 7 shows the separation of two proteins, phycoerythrin and phycocyanin the latter of which the molecular weight is 150,000 in a similar system. Thus it appears to be possible to carry out counter-current distribution of large molecular weight protein in aqueous polymer phase system.

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ON THE FUNCTIONS OF THE ENDOPLASMIC RETICULUM

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Abstract—Ribonucleoprotein (RNP) particles were purified in a highly active state from a number of tissues of the rat. Particle yield varied considerably for different tissues but the composition of particles was fairly similar. From the early blastula stage of *Psammechinus* eggs active particles were also obtained, and at a higher yield than from preceding stages of development. Mitochondria also yielded protein synthesizing particles in a study of other cell components. No significant amino acid incorporation was obtained with purified RNP particles when they were used alone. Mechanisms of amino acid incorporation are discussed. Experiments show the possibility of a functional interrelationship between endoplasmic particles and membranes.

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A GOOD deal of evidence has accumulated in the last few years in favour of the idea that ribonucleoprotein particles (RNP-particles) are intimately involved whenever proteins become synthesized in the cytoplasm. These particles may occur in a free state, but they are usually associated with membranous structures of some kind. The majority of them are attached to the so-called *endoplasmic reticulum*, a system of submicroscopic ducts and vesicles, by which the cytoplasm is penetrated. Fragments of this reticulum can be isolated from tissue homogenates by differential centrifugation, becoming concentrated in the so-called *microsomal fraction*.

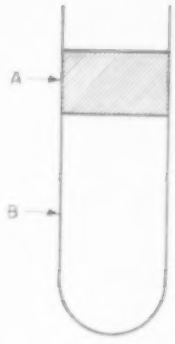
A little more than 10 years ago we observed that in liver the microsomal material was characterized by an extraordinarily rapid protein metabolism.[1] It has subsequently been shown, particularly by Zamecnik and his group in Boston, that under suitable conditions also isolated microsomes can give rise to an incorporation of labelled amino acids into protein.[2, 3]

It has been demonstrated in several different ways that the nucleoprotein constituents of the microsomal material are primarily involved in the incorporation reaction.[4–8] In spite of this fact, attempts to obtain amino acid incorporation *in vitro* by RNP-particles, free from adhering membranes, have not been very successful. Recently, however, a method has been worked out in our laboratory by Rendi and myself, by which it has been possible to prepare relatively pure RNP-particles in a highly active state (Fig. 1).

By use of this method RNP-particles have been prepared from a number of rat tissues. As may be anticipated, the yield of particles varied considerably from one kind of tissue to another. The composition of the particles, however, was fairly similar, with ribonucleic acid (RNA) contents in some cases approaching 40 per cent (Table 1). The capacity of these different kinds of particles to incorporate labelled amino acids

in vitro was also very much the same, when measured on the basis of equal RNA content (Table 2).

Particles have been prepared in this way not only from mammalian tissues but also from sea urchin eggs and embryos. These particles, also, proved quite active in incorporation systems. When particles were isolated from equal samples of embryos, taken



A: Mitochondria-free homogenate in:
0·15 M sucrose
0·025 M KCl
0·01 M $MgCl_2$
0·035 M tris pH 7·8 — 6·5 ml

mix with: (1) 2·5 M KCl in 0·01 M $MgCl_2$ — 2·0 ml
(2) 10% Lubrol W in 0·01 M $MgCl_2$ — 0·5 ml
(3) 10% deoxycholate — 1·0 ml

B:
0·3 M sucrose
0·6 M KCl
0·01 M $MgCl_2$
0·035 M tris

FIG. 1. Procedure for the preparation of active RNP-particles from liver microsomes.

at different periods from a developing culture, the total activity of the preparations for amino acid incorporation rapidly increased in the course of the early blastula stage (Table 3). The hypothesis has previously been advanced that populations of protein-synthesizing particles gradually develop in the cytoplasm of the early blastula cells in connexion with the primary determination, the process by which the extension of the main embryonic regions becomes irreversibly established.[9, 10] The present results seem to confirm this hypothesis.

TABLE 1. PROTEIN AND RNA CONTENT OF FRACTIONS OF RNP-PARTICLES, PREPARED FROM DIFFERENT TISSUES OF THE RAT BY THE METHOD ILLUSTRATED IN FIG. 1

Tissue	Fresh tissue (mg/g)		RNA %
	RNA	protein	
Liver	0·84	1·38	38
Kidney	0·46	1·46	24
Spleen	0·74	1·00	41
Brain	0·20	0·41	33
Testis	0·40	0·61	39

The method illustrated by Fig. 1 has been used for the preparation of RNP-particles not only from the endoplasmic reticulum but also from other cell components. In this way Rendi in our laboratory has obtained good evidence for the presence of such protein-synthesizing particles in mitochondria.[11] It may be justifiable, therefore, to propose that we are dealing here with a sort of biological elementary particles, universally responsible for the cytoplasmic production of cell proteins.

No significant amino acid incorporation is obtained, however, with the purified RNP-particles alone, since these have no (or very small) ability to activate the amino acids. I feel that the already classical amino acid activating enzymes of Hoagland[12], which mainly occur in the so-called pH 5 fraction of the cell sap, are too well known at present to be more than briefly discussed in this connexion. As is well known, these

TABLE 2. INCORPORATION OF LABELLED AMINO ACIDS INTO PROTEIN BY RNP-PARTICLES PREPARED FROM DIFFERENT TISSUES OF THE RAT

(The incubation mixture contained: 0·08 μmoles of ^{14}C-amino acids, 1 μmole of ATP, 10 μmoles of phosphoenolpyruvate, rat liver supernate, containing 2 mg of protein, and RNP-particles prepared from different tissues and containing approximately 2mg of RNA.)

Tissue	Total counts incorporated	
	^{14}C-L-leucine	^{14}C-L-valine
Liver	3700	6200
Kidney	2700	—
Brain	2400	4800
Spleen	2100	3700
Testis	3000	4000

TABLE 3. INCORPORATION OF ^{14}C-L-LEUCINE INTO PROTEIN BY FRACTIONS OF RNP-PARTICLES PREPARED FROM *Psammechinus* EGGS AT DIFFERENT STAGES OF DEVELOPMENT

(The fractions of particles were incubated in a system of the same kind as in Table 2, with the difference, however, that the samples of added liver supernate contained about 10 mg of protein.)

Particles prepared from	Incorporation (counts/min per cm^2)
Unfertilized eggs	45
Fertilized eggs, 30 min	58
Blastulae, before hatching	275

enzymes (E) are able to activate amino acids (aa) by splitting off pyrophosphate (PP) from adenosine triphosphate (ATP):

$$\text{aa} + \text{ATP} + \text{E} \rightleftharpoons \text{E} - \text{AMP} \sim \text{aa} + \text{PP} \qquad (1)$$

The reaction is reversible, and in the presence of amino acids an isotope exchange between added ^{32}P-pyrophosphate and ATP is catalysed by the enzymes. This pyrophosphate exchange reaction can easily be measured, and it has frequently been used both for qualitative and quantitative studies of the activation reaction.[12–16] It has been demonstrated by experiments of this kind that separate enzymes are responsible for the activation of different amino acids, although some overlapping may be observed under certain conditions.[12–14, 17–21]

By the activation reaction adenylates of the amino acids are formed.[17, 19, 20] Amino acid adenylates have been prepared also by chemical synthesis.[17, 22, 23] Since these

compounds have the chemical character of anhydrides, they are highly reactive and spontaneously transfer the amino acid residue to acceptor molecules of different kinds, even proteins.[20, 22] However, no such spontaneous transfer of labelled amino acids to protein takes place in the enzymic activation systems, and no traces of free adenylates occur.[23] The adenylates, therefore, must be present only in catalytic amounts, firmly bound to the enzymes.

We observed some years ago that the incorporation of labelled amino acids into protein by cytoplasmic systems is accomplished by two successive steps:[24]

(1) In the presence of the soluble fraction and a suitable energy supply a derivative of the amino acid accumulates in the system, without equilibrating very rapidly with the free amino acid pool.

(2) When microsomes are then added, the amino acid is directly transferred from this intermediate derivative and incorporated into protein (Fig. 2). It has been shown

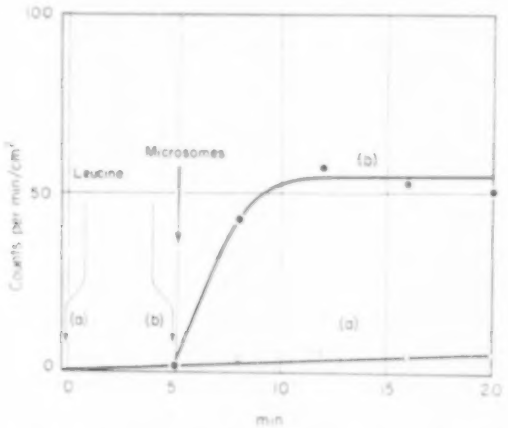


FIG. 2. The incorporation of ^{14}C-L-leucine into protein by a rat liver system, visualized as a two-step reaction. The incubation started with a system containing 0·6 ml of cell sap, 0·02 μmoles of ^{14}C-L-leucine, 1 μmole of ATP and 10 μmoles of phosphoenolpyruvate. A 1000-fold isotope dilution was effected by the addition of 20 μmoles of L-leucine (a) at zero time, (b) after 5 min of incubation. In both cases microsomes were added after 5 min of incubation. No significant incorporation was obtained without added microsomes. Trichloroacetic acid was added to the tubes at the periods indicated.

by the Boston workers[25] that the intermediate amino acid carrier is a ribopolynucleotide, to which the amino acid is specifically transferred from the enzyme-bound adenylate:

$$E - AMP \sim aa + polynuc \rightleftharpoons (E-)\ polynuc - aa + AMP^{26} \qquad (2)$$

The polynucleotide is normally associated with the enzyme.[25, 27] It can be isolated, however, from the pH 5 fraction, and purified without losing its activity as amino acid carrier in the incorporation system.[25, 28] There is good reason to believe that the interaction between a polynucleotide of this kind and the corresponding enzyme is specific, and that different kinds of polynucleotides act as carriers for different kinds of amino acids.[28, 29]

When a purified polynucleotide preparation carrying a labelled amino acid is incubated with microsomes or with RNP-particles, the amino acid becomes incorporated into protein. For this to happen, however, the system must contain: (1) a proper energy-generating system; (2) guanosine triphosphate; (3) a soluble enzyme preparation:

$$(E-)\ \text{polynuc} - \text{aa} + \text{RNP} - \text{protein} \underset{?}{\overset{\text{GTP, enzyme}}{\rightleftharpoons}} \text{RNP} - \text{protein} - \text{aa} + ? \quad (3)$$

A little more than 2 years ago we became interested in the question: what happens with the carrier polynucleotide itself in the course of this final incorporation step? Some isotope experiments, carried out by von der Decken and myself[30, 31] pertain to this question. We observed that under the same exacting conditions (cf. above) required so that the nucleotide-bound amino acid could become incorporated into protein by a RNP-particle, the *polynucleotide itself became associated with the particle* (Tables 4 and 5).

TABLE 4. REQUIREMENTS FOR ISOTOPE TRANSFER FROM SOLUBLE POLYNUCLEOTIDES TO MICROSOMAL NUCLEOPROTEIN BY A RAT LIVER SYSTEM

(The prelabelled soluble fraction had been dialysed for 20 hr. The microsomes had been prepared from non-labelled, regenerating liver. The tubes (final volumes 5·0 ml) were incubated for 8 min at 35 °C, except the control tubes, which were left in the ice-bath. PEP = phosphoenolpyruvate (30 μ moles); ATP, GTP, UTP, CTP = adenosine, guanosine, uridine and cytidine triphosphate (3 μ moles))

	Supernatant prelabelled with			
	^{32}P-phosphate		^{14}C-adenine	
	Counts/min per 100 μg phosphate in		Counts/min per mg RNA	
	Cytidylic acid	Uridylic acid		
Microsomes, labelled supernatant			21,	11
plus ATP	230	260		
PEP	130	220		
PEP, ATP	870	800	51,	35
PEP, ATP, amino acids	750	830	52	
PEP, ATP, GTP, UTP	960	915		
PEP, ATP, GTP, UTP, amino acids	970	930		
PEP, ATP, GTP, UTP, CTP				43
PEP, ATP, GTP, UTP, CTP, amino acids			58,	46
kept in ice-bath during incubation	200	360	17,	14

It is commonly believed that the function of RNA in protein synthesis is to arrange the different amino acids in proper order before they become incorporated into a developing peptide chain. Chemically, however, it is difficult to understand, how a RNA-molecule could act directly upon individual amino acids in this highly discriminating way. The experiments just mentioned suggest that specific carrier-polynucleotides may pilot the amino acids into their proper sites on the RNA-"template" by *becoming associated themselves* with identical or complementary nucleotide

sequences along the RNA-molecule. We have often tried to illustrate this idea by the scheme shown in Fig. 3. On the basis of such mutual interactions between nucleotide sequences alone it seems possible to give the template function in protein synthesis a chemically intelligible interpretation. Evidence in favour of a "coding" mechanism of this kind has been presented also by Hoagland[32].

The amino acids are bound to the polynucleotide carriers by ester linkages with relatively low energy content.[28, 33] The function of guanosine triphosphate in the final

TABLE 5. REQUIREMENTS FOR ISOTOPE TRANSFER FROM SOLUBLE POLYNUCLEOTIDES TO MICROSOMAL RNP-PARTICLES BY A RAT LIVER SYSTEM

(The incubation system contained in final volumes of 3 ml : 200 μg of labelled, purified polynucleotides, microsomes corresponding to 2·5 g of fresh liver, 120 μg of pyruvate kinase, 200 μg of myokinase. Mg-concentration 0·005 M. After the incubation RNP-particles were isolated.)

	Counts/min per mg ribonucleotide
Microsomes, ^{32}P-labelled polynucleotide plus PEP, ATP, GTP	255
supernatant, GTP	110
ATP, GTP	120
PEP, GTP	205
PEP, ATP, GTP	330
PEP, ATP, GTP	130*
pH 5 fraction, PEP, ATP	195
PEP, ATP, GTP	310

* Kept in ice bath during incubation.

incorporation step (3) may be to reactivate the amino acid, possibly by forming an amino acid guanylate capable of immediate reaction *in situ* with the neighbouring amino end group of the developing peptide chain. The specificity may thus be gained in the incorporation process at the expense of a repeated energy investment.

It is only possible to speculate, at present, about the ultimate fate of the carrier polynucleotide. One possible inference of the polynucleotide transfer (Tables 4, 5) may be of some interest, however, for the debate on the postulated self-reduplication of the RNP-particles. When the carrier polynucleotides are stripped of their amino acids at the moment of incorporation, the polynucleotide residues themselves may become linked together under formation of a new RNA-molecule. This possibility is consistent with a number of isotope experiments *in vivo*, indicating that polynucleotides in the cell sap become labelled earlier than the RNA of the particles.[34–36]

It has been shown by Sachs[37] that labelled amino acids may become incorporated into protein by liver microsomes under the influence of a polynucleotide-free preparation of soluble enzymes. This preparation ("s-protein") has been further studied in Campbell's laboratory,[38] and it has recently been somewhat purified by Rendi and myself[39]. The purified s-protein enhanced the amino acid incorporation both by microsomes and by isolated RNP-particles. In spite of this, only a small amino acid dependent pyrophosphate exchange was catalysed by this enzyme preparation, and it could be demonstrated by ^{32}P-labelled preparations that no nucleotides were present.

A characteristic property of the s-protein is its marked sensitivity to the redox level of the medium. Its action becomes greatly stimulated by sulphydryl compounds like mercaptoethylamine and inhibited by oxidative agents (Table 6). As a rule, the amino acid in corporation in the presence of s-protein became stimulated by added polynucleotide. Whether polynucleotide was added to the system or not, the amino acid incorporation by s-protein was strongly inhibited by ribonuclease, and guanosine triphosphate could be shown to stimulate the incorporation process. This seems to indicate

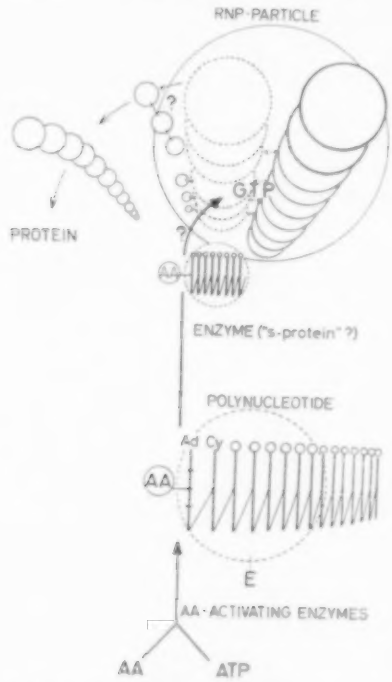


FIG. 3. Diagram representing the conception of the incorporation process, underlying the polynucleotide transfer experiments mentioned in the text. The carrier-polynucleotides (symbolized by dotted circles) are supposed to become specifically attracted by identical or complementary nucleotide sequences within the RNA-template (system of continuous circles). The incorporation by the s-protein system may occurr independently of the polynucleotide mechanism. Since the incorporation is strongly inhibited by ribonuclease, the template-RNA may participate in the reaction. It is difficult, however, at present to visualize how this may occur.

that the two kinds of incorporation systems discussed here, the system with particles and pH 5 fraction and the system with particles and purified s-protein, have at least the final enzymic path in common (Fig. 3).

The problem arises, whether purified RNP-particles are capable of synthesizing specific, fully differentiated proteins. This question seems particularly justified in the case of the incorporation systems with particles and purified s-protein, freed from carrier polynucleotides. We are trying at present to answer this question by studying the ability of different systems to incorporate labelled amino acids, not only into acid-precipitable protein matter but into specific, characterizable species of protein molecules.[40–43]

Two different incorporation systems have been tried so far:

(1) The incorporation of labelled amino acids into haemoglobin by reticulocyte particles.[41]

(2) The incorporation of labelled amino acids into serologically characterizable protein antigens by means of microsomes or RNP-particles from liver.[42, 43]

The experiments, which are carried out by Morgan, von der Decken, Perlmann and myself, are still in a preliminary state. So far, no incorporation of ^{14}C-L-leucine into

TABLE 6. EFFECTS OF POLYNUCLEOTIDE AND REDUCING AGENTS ON THE INCORPORATION OF ^{14}C-L-LYSINE INTO PROTEIN BY RNP-PARTICLES AND S-PROTEIN

(The incubation system contained in 1 ml, 0·08 μmoles ^{14}C-L-lysine, 1 μmole ATP, 10 μmoles phosphoenolpyruvate. MEA = mercaptoethylamine; DPN, DPNH = diphosphopyridine nucleotide, oxidized and reduced; TPN, TPNH = triphosphopyridine nucleotide, oxidized and reduced.)

	Counts/min per cm^2
Controls	67, 69
with MEA (12 μmoles)	218
polynucleotide (50 μg)	136
MEA + polynucleotide	273
DPNH (2 μmoles)	97
DPN (2 μmoles)	56
TPNH (2 μmoles)	93
TPN (2 μmoles)	63

specific proteins has been observed with certainty in the absence of carrier nucleotides, but the experiments are being continued with other amino acids.

As was mentioned before, the RNP-particles are generally attached to the membranes of the endoplasmic reticulum. The fact that such RNP-particles, which have been freed from the membranes, are also able to incorporate labelled amino acids into protein, does not mean that the membrane material is without any influence on the incorporation process under physiological conditions. This fact became particularly obvious in the incorporation experiments with subcellular preparations from sea urchin eggs. It was observed that total homogenates or mitochondria-free extracts from fertilized eggs had a much higher ability to incorporate ^{14}C-L-leucine into protein than the corresponding preparations from the unfertilized eggs. In parallel experiments with isolated RNP-particles this difference was less significant. It is tempting to assume that the endoplasmic membranes in the preparations were responsible for the greatly reduced incorporation capacity before fertilization.

We have observed that in liver the ability of the microsomes to incorporate labelled amino acids into protein may become influenced by enzymatic processes taking place in the membranes. It has been shown that a number of oxidative and reductive reactions, related to metabolic detoxication, are catalysed by enzymes in the endoplasmic membranes.[44–48] Under certain conditions such reactions may diminish the activity of the particles in incorporation systems. This has been observed in experiments with the water-soluble liver carcinogen dimethylnitrosamine,[49–50] and further examples of inhibitions of this kind by liver carcinogens and by other compounds

metabolized by the endoplasmic membranes of the liver have been presented by Arrhenius and myself[51] previously at this meeting.

The experiments discussed here indicate the possibility of a functional interrelationship between the endoplasmic particles and membranes. It is generally assumed that the rate of protein metabolism in a tissue cell is determined under the influence of manifold, integrated regulation factors. A factor of supreme importance for the total capacity of protein synthesis may obviously be the number of RNP-particles. As was mentioned before an increased rate of protein metabolism due to a higher content of active RNP-particles can be demonstrated in sea urchin embryos (Table 3 [10]), and a similar situation has previously been observed in regenerating rat liver.[15] It is our feeling that the particle–membrane interrelationship in the endoplasmic reticulum may affect the level of protein metabolism in a more direct and flexible way.

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Biochemical Pharmacology, 1961, Vol. 5, pp. 369–380. Pergamon Press Ltd., Printed in Great Britain.

ON SUBCELLULAR ELECTRON TRANSPORT PHOSPHORYLATION SYSTEMS*†

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Abstract—Adenosine triphosphate is known to be a universal carrier of chemical energy between the energy-yielding and energy-requiring processes of living cells. In chlorophyll-containing organisms, such as green plants and photosynthetic bacteria, light induces a phosphorylation of adenosine diphosphate to adenosine triphosphate. The fact that this formation of adenosine triphosphate in the chloroplasts of the plants and the chromatophores of the bacteria is linked to electron transport invites comparison with oxidative phosphorylation in mitochondria. The biochemical mechanisms of oxidative and light-induced phosphorylation are discussed. New evidence is given for certain similarities and some differences between the mechanisms of electron transport phosphorylation in the various systems. The electron transport and phosphorylation reactions involved are not yet completely understood. It appears useful to base any working hypothesis for the investigation of electron transport phosphorylation in various sub-cellular systems on the established occurrence of similarities. Methods applied and results obtained with any of the three well-defined sub-cellular electron transport phosphorylation systems appear to become increasingly valuable when the other ones are being investigated.

INTRODUCTION

ABOUT a half century ago, there existed a rather common opinion, that the phenomenon of life constitutes a revolution against the second law of thermodynamics. This law states that the total amount of entropy in Nature is increasing, or, in other words, that organization goes towards a minimum. Today it is generally recognized that the revolution was only fictional. Life is a "steady state" system and, both when taken as a whole and when it is represented in a single organism, it does not have the properties of a closed system in equilibrium where the basic laws of thermodynamics apply. Life as we define it today ultimately depends on the radiation which the earth is receiving from the sun. Chlorophyll-containing organisms in the reactions of photosynthesis use energy from this radiation to build complicated organic molecules, where part of the energy consumed for their biosynthesis is conserved. Thus the utilizable energy content of these molecules is much higher than that of their primary building stones, water and carbon dioxide. In the reactions where the organic molecules are built, an essential role is played by adenosine triphosphate (ATP).

All available evidence indicates that ATP is the universal, primary carrier of chemical energy between the energy-yielding and energy-requiring processes of living

* "Electron transport phosphorylation" will be used throughout to denote phosphorylation linked to electron (or hydrogen) transport along a chain of electron carriers.

† Abbreviations used in this paper are: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; FMN, flavin mononucleotide; FAD, flavinadenine dinucleotide; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline-N-oxide.

cells. Thus, as soon as the light quanta from the radiation of the sun have given away their energy to the chlorophyll-containing organism in a photophysical or photochemical reaction, this energy must be transferred through a suitable metabolic system to ATP in order to be useful to the organism. As early as 1943 Ruben[1] suggested that coupled oxido-reduction following the photochemical reaction could convert radiant energy to "high energy phosphate donors".

Recent research has shown that an ATP-generating system, closely linked to the photophysical or photochemical reaction, indeed exists. The formation of ATP is linked to electron transport. The process was found in 1954 by Arnon *et al.*[2] in isolated spinach chloroplasts and by Frenkel[3] in extracts of photosynthetic bacteria. It was given the name photosynthetic[2] or light-induced[3] phosphorylation.

Both in bacteria and in plants the system for light-induced phosphorylation is localized in sub-cellular, chlorophyll-containing structures: chromatophores in bacteria and chloroplasts in plants. Both in bacteria[4] and in plants[5] adenosine diphosphate (ADP) is phosphorylated to ATP in the energy-trapping reactions. This resembles oxidative phosphorylation of mitochondria. How close this resemblance appears to be and what similarities and differences the bacterial and the plant systems for light-induced phosphorylation show, when compared with each other, will be the subject of this lecture.

One could divide the items to be compared into three categories: structure of the particles, mechanism of the electron transport, and mechanism of the phosphorylation reactions. Only the two first groups will be dealt with in some detail, as knowledge about the third is still very limited. The reason why light-induced phosphorylation in plant chloroplasts and bacterial chromatophores will be compared with oxidative phosphorylation in animal mitochondria, and not in plant mitochondria or bacterial structures, is that most of the available information about mitochondria and oxidative phosphorylation has been obtained with animal material. Functional properties of the systems for light-induced phosphorylation will be represented by those from the best known sources in this respect: the photosynthetic bacterium *Rhodospirillum rubrum* in the case of bacteria and spinach in the case of plants.

SUB-CELLULAR STRUCTURES FOR ELECTRON TRANSPORT PHOSPHORYLATION

Three different sub-cellular sites for electron transport phosphorylation, namely, animal mitchondria, plant chloroplasts and bacterial chromatophores, will be briefly described and compared. Our knowledge about them is derived mainly from electron microscopic and biochemical studies.

Animal mitochondria are usually somewhat elongated *in vivo*. A normal size of liver and heart muscle mitochondria is approximately 0·5 by 2 μ. They are surrounded by a double membrane[6] and reveal more or less densely packed, parallel internal double membrane structures.[6, 7] Each double-membrane is considered to consist of two protein layers and a lipid layer. The electron transport chain is intimately connected with the membranes. The electron carriers appear to be oriented in the membranes and arranged in functionally suitable "assemblies"[8] or a network of "sub-units".[9] From the mitochondria it has been possible to isolate components of the electron transport chain as free coenzymes, as proteins and as lipoproteins. Oxidative phosphorylation has been obtained in suspensions of sub-mitochondrial particles (from

isolated mitochondria) but not in solution. The phosphorylating sub-mitochondrial fragments described by Cooper *et al.*[10] were reported to have a particle weight of 50 million.

Plant chloroplasts are lamellar bodies of varying size and shape. They can be several microns in diameter. In higher plants the lamellae are differentiated into the non-pigmented stroma lamellae and the more or less cylindrical, pigmented, chlorophyll-containing grana lamellae. The lamellar bodies appear to consist of alternating lipophilic (lipid-containing) and hydrophilic (protein-containing) layers of a double membrane[11] nature. The lipid- and protein-containing layers have been proposed to be separated by monomolecular films of chlorophyll molecules, with their lipophilic phytol chain reaching into the lipid layer.[12] The chlorophyll content of chloroplasts on a dry weight basis is 5–6 per cent. A minimal functional sub-unit in the chloroplast has been suggested.[13] It would contain 100–300 chlorophyll molecules. Two compounds capable of undergoing oxidation and reduction which are found in chloroplasts, and to which an electron transport function has been ascribed, have been quantitatively determined in relation to chlorophyll. They are vitamin K_1 and cytochrome *f*. The ratio of chlorophyll to vitamin K_1 is roughly 250[14, 15] and chlorophyll to cytochrome *f* about 170, according to a recent estimation.[16] Thus a possibility exists that every proposed photosynthetic sub-unit of the chloroplast is structurally and functionally related to a single electron transport chain.

Bacterial chromatophores are comparatively small organelles. Those of *Rhodospirillum rubrum* have an estimated diameter of 400 Å and a tentative "molecular weight" of 30 million.[17] A single cell is reported to contain about 5000–6000 chromatophores which seem to be evenly distributed throughout the cell. It is still a matter of disagreement whether the bacterial photosynthetic apparatus contains lamellar structures[18, 19] or not.[20] According to Newton and Newton[21] *Chromatium* chromatophores contain 200 molecules of chlorophyll–chromatophore, and the ratio chlorophyll–carotenoid–cytochromes is 10 : 5 : 1. The figure 200 invites comparison with the figures 100–300 in the postulated chloroplast sub-unit.

As an example of the difference in size between chloroplasts of algae and higher plants and bacterial chromatophores, *Euglena* chloroplasts are from two to three times the size of the whole bacterium *Rhodospirillum rubrum*. As Kamen and Newton[22] have pointed out, one may regard a photosynthetic bacterium as analogous to a chloroplast and the chromatophores as analogous to the chloroplast grana. A chromatophore may perhaps also be compared with the postulated sub-unit of the grana membranes.

In summary, the comparison between the structures of mitochondria, chloroplasts and chromatophores shows certain similarities. Present knowledge allows only a dim perception of the details of the various structures. In the search for common denominators one should not neglect the major functional difference between chloroplasts and chromatophores, which transform light energy to chemical energy, and mitochondria, where respiration and oxidative phosphorylation seem to be the main function. In a symposium such as this, however, one may perhaps be allowed to speculate about why electron transport phosphorylation is confined to particles. Oriented double membranes which provide both hydrophilic and lipophilic phases dominate the pictures of mitochondria and chloroplasts, and have been suggested to occur in the chromatophores also. As will be discussed below, both hydrophilic and lipophilic compounds seem to take part in electron transport phosphorylation. Consequently

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the phase boundary between the lipophilic and hydrophilic phase can be assumed to contain the electron transport phosphorylation system. The reason why particles are necessary may be that they can provide this boundary between two phases. Assuming that great boundary surface means great capacity for electron transport phosphorylation, one finds that the achitecture of the particles is remarkably rational.

ELECTRON TRANSPORT AND PHOSPHORYLATION

Oxidative phosphorylation in animal mitochondria

An outline of what is known at present about electron transport and oxidative phosphorylation in animal mitochondria will now be given. It is not possible here to follow in any detail the fascinating development of the concept of an electron transport chain for the respiration of the cell. The picture of a chain emerged mainly through the investigations of Keilin and Warburg and their co-workers in the nineteen twenties and thirties. Warburg[23] wrote in 1934 about the function of such a chain: "Es liegt nahe anzunehmen, dass die Aufteilung der Energie der Atmung in kleinere Teile günstig ist für das, was die Natur mit der Atmung bezweckt: die Verwandlung chemischer Energie in Arbeit". Today one would change the six last words to "synthesis of ATP, where chemical energy is preserved".

There are still different opinions about details of the respiratory chain in oxidative phosphorylation. Each year new information is added. Fig. 1 may be said to represent my concept of it today. It is based mainly on the schemes of Slater[24] and of Chance and Williams[25] and on recent results published by Hatefi[26].

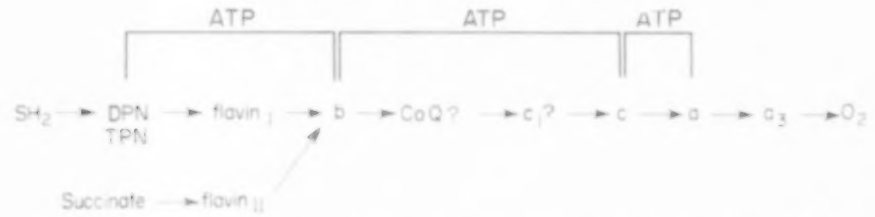


FIG. 1. Respiratory chain and regions of phosphorylation in animal mitochondria. Abbreviations: SH_2 = substrate, *b*, c_1 etc., = cytochrome *b*, cytochrome c_1, etc.

As is seen, pyridine nucleotide accepts hydrogen from the substrates, except from succinate, which directly reduces a flavoprotein, succinic dehydrogenase.[27] DPNH reduces another flavoprotein. TPNH has been reported to become oxidized in phosphorylating mitochondria along two different pathways,[28] over DPN through the action of pyridine nucleotide transhydrogenase and over TPNH-oxidase. The two branches of the respiratory chain join at cytochrome *b* and from here on the electrons flow, as is indicated in Fig. 1, along a single chain to reduce the final acceptor, oxygen. A quinone, coenzyme Q, is reported to be situated between cytochromes *b* and *c*.[26] Vitamin K[29, 30] and vitamin E[31] have been suggested as links in the phosphorylating electron transport chain, but the situation with respect to these agents seems less clear. It may be noted that they both are also quinones. Cytochrome c_1 participates in electron transport in non-phosphorylating mitochondria,[32] but a role for it in oxidative phosphorylation has not been demonstrated.

To sum up, there may be four different types of compounds carrying electrons from substrate to oxygen in animal mitochondria: pyridine nucleotides, flavoproteins, cytochromes and quinones.

Although many attempts have been made during the last ten years to clarify the mechanism of oxidative phosphorylation it is essentially still obscure. Several general or specified mechanisms have been suggested[25, 33–38] but a detailed and comprehensive logical picture is lacking. Thus it may suffice here to mention a few generally accepted facts.

(1) Owing to the flow of two electrons between DPNH and O_2, three molecules of ATP are formed. Two are formed between succinate and O_2, two between DPNH and cytochrome *c* and one between cytochrome *c* and O_2.

(2) The normal potentials of the redox systems involved are such that the above values are energetically feasible.

(3) When the electrons flow in the electron transport chain from a higher energy level to a lower, they give off potential energy. This energy is utilized to link inorganic phosphate by a "high energy bond" with an intermediate phosphate acceptor. The product reacts with ADP to give ATP.

Light-induced phosphorylation in bacterial chromatophores and plant chloroplasts

Light-induced phosphorylation has been shown to be linked to electron transport, for example by means of its stimulation by electron carriers and inhibition by typical inhibitors of electron transport. In the presence of light, photosynthetic systems are capable of producing a reductant and an oxidant. Light-induced phosphorylation is generally pictured as resulting from the reaction of these with each other over a chain of electron carriers. The concept of an electron transport chain in light-induced phosphorylation immediately suggested the possibility that similiarities existed between this system and that of mitochondrial oxidative phosphorylation. Current views of electron transport in light-induced phosphorylation will now be presented, and after this some of our own results will be demonstrated and discussed.

The result reported by Smith and Baltscheffsky[39] that 2-*n*-heptyl-4-hydroxyquinoline-N-oxide (HOQNO) completely inhibited light-induced phosphorylation in extracts of *Rhodospirillum rubrum* indicated that the process in bacteria was linked to electron transport. The inhibitor is known to block the electron transport in animal mitochondria between cytochrome *b* and cytochrome c_1.[40] The assumption that the site of action is identical or very similar in the bacterial extracts is supported by the finding of Geller[41] that also antimycin A and SN 5949 (a naphthoquinone) which act at the same level as HOQNO in mitochondrial electron transport inhibit the bacterial system. Further support for similarity between the two systems was obtained when Smith and Baltscheffsky,[42] working with both whole cells and extracts of *R. rubrum*, found that HOQNO caused a reduction of a *b*-type cytochrome and an oxidation of cytochrome c_2, as compared with the uninhibited systems. This finding demonstrated participation of cytochromes in the electron transport reactions of light-induced phosphorylation in bacteria. The following scheme was given to account for the results:

"photochemical apparatus"

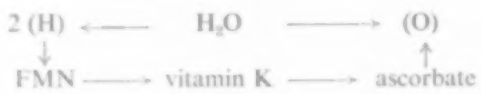
↓ ↓

reductant → cytochrome chain including cytochrome c_2 → oxidant
and probably a *b*-type cytochrome

Up to now it is the most detailed scheme given for electron transport in light-induced phosphorylation of bacteria. On the basis of the above-mentioned studies, Frenkel has also included cytochrome c_2 in a schematic representation of electron transport in light-induced phosphorylation of *R. rubrum* chromatophores[43] (cf. Frenkel[44]).

Several electron carriers have been suggested as physiological co-factors in light-induced phosphorylation of plants. In contrast to the chromatophores of *R. rubrum* where high rates of phosphate esterification have been obtained without addition of external electron carriers, isolated plant chloroplasts have given only extremely low rates of light-induced formation of ATP in the absence of added electron carriers. In the presence of agents such as menadione,[45] FMN[46] or phenazine methosulphate[47] very high rates of phosphorylation have been obtained.[48] When considering electron transport properties in light-induced phosphorylation of isolated spinach chloroplasts it must be kept in mind that the presence of an added carrier may possibly introduce a by-pass for the electrons around a smaller or greater portion of a physiological electron transport chain.

In 1955 Arnon *et al.*[45, 48] found that ascorbate, flavin mononucleotide and menadione stimulated light-induced phosphorylation in isolated spinach chloroplasts. They suggested that the conversion of light energy into the pyrophosphate bond energy of ATP involved an electron transport chain according to the following tentative scheme:



In this scheme, water was photolysed in the Hill reaction[49] to a reductant and an oxidant, the electron transport chain transporting electrons from the former to the latter. Owing to the intensive subsequent research by these and other workers the scheme is today, 4 years later, only of historical interest. It is only possible here briefly to mention some of the important recent developments. Arnon *et al.*[50] have found that the rate of ATP-formation in the presence of light can be stimulated by TPN. Their conclusion that TPN participates in the electron transport of the process is, however, not shared by Wessels.[51] Wessels[52] and Whatley *et al.*[53] agree that flavin mononucleotide and menadione appear to catalyse separate pathways of electron transport in light-induced phosphorylation and that ascorbate may act primarily as a protective agent for the chloroplasts.[51, 53] According to Jagendorf and Avron[47] flavin mononucleotide and menadione as well as phenazine methosulphate may all act by bridging a gap in the electron transport of the chloroplasts, which may have been damaged during their isolation.

In a very recent article on photosynthesis Arnon[54] suggests that ATP can be formed in the presence of light in chloroplasts of green plants along three different pathways: (1) the cyclic electron transport system involving vitamin K and a cytochrome, (2) the cyclic electron transport system involving TPN, flavin mononucleotide and cytochromes, and (3) the "open" non-cyclic mechanism, which transports electrons from chlorophyll to TPN. In (3), molecular oxygen is produced in an equivalent amount to the TPN reduced. In this system or in model experiments, where ferricyanide[55] is substituted for TPN, one molecule of ATP is formed for each two electrons reaching the acceptor. A different sensitivity towards various inhibitors (*o*-phenanthroline,

2 : 4-dinitrophenol) and towards chloride ion has prompted the postulation of the separate electron transport chains.

Practically nothing is known about the mechanism of the phosphorylation reactions in light-induced phosphorylation. Mg^{2+}-ion is a necessary co-factor for the formation of ATP both in chromatophores[4] and chloroplasts.[56] In isolated chloroplasts no ATPase reaction or exchange reaction between ATP and ^{32}P was found by Avron and Jagendorf,[57] who took this as evidence that the last phosphorylation reaction is irreversible. On the other hand, we have shown that a Mg^{2+}-stimulated ATPase activity exists in isolated spinach chloroplasts.[58] Oligomycin A, which appears to act on a phosphate transfer reaction in mitochondrial oxidative phosphorylation,[59] inhibits strongly light-induced phosphorylation in bacterial chromatophores,[60] but

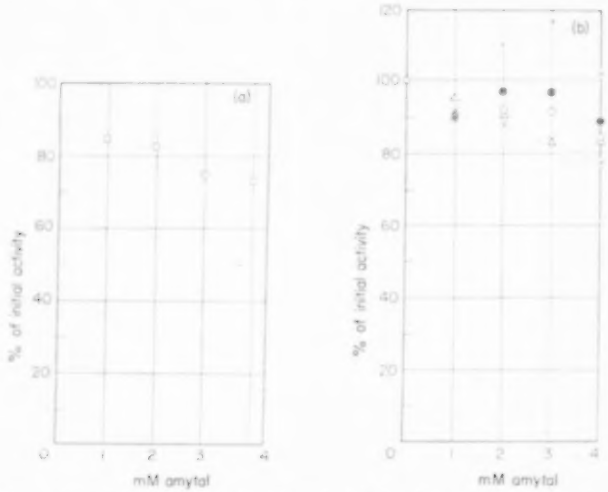


FIG. 2. Effect of amytal on light-induced phosphorylation in chromatophores and chloroplasts. (a) Chromatophores; experimental details as Baltscheffsky[72]. (b) Chloroplasts. The following stimulating agents were used: × = FMN, ○ = FAD, △ = phenazine methosulphate, ● = menadione and + = ascorbate. The phenazine methosulphate and menadione series represent mean values of three and two experiments, respectively. The rate of phosphorylation in the ascorbate series was extremely low. With the exception of the phenazine methosulphate series all tubes contained 30 μmoles of ascorbate. Further experimental details as in Baltscheffsky[61]. Partially reproduced from *Acta Chem. Scand.*[61].

has very little effect in plant chloroplasts.[61] This may indicate that the phosphorylation mechanisms are different in these two systems.

Recently we have obtained some new information about the electron transport in light-induced phosphorylation using certain well-known inhibitors of electron transport in phosphorylating animal mitochondria. The inhibitors employed were amytal, atebrin, antimycin A and HOQNO. In mitochondria, amytal acts between diphosphopyridine nucleotide and flavin,[62, 63] atebrin at the flavin level[64, 65] and antimycin A and HOQNO, as mentioned, between cytochrome *b* and cytochrome c_1.

As is shown in Fig. 2 amytal did not inhibit light-induced phosphorylation in either chromatophores* or chloroplasts. This suggests that photosynthetic systems lack an

* Mrs. M. Baltscheffsky participated in most of the experiments with photosynthetic bacteria.

electron transport step identical with that between DPNH and flavin in phosphorylating mitochondria. Earlier it has been shown that diphosphopyridine nucleotide does not seem to be involved in light-induced phosphorylation of chloroplasts.[50]

Low concentrations of atebrin strongly inhibited light-induced phosphorylation in extracts of *Rhodospirillum rubrum*. This is shown in Fig. 3, where it can also be seen that FAD is capable both of stimulating markedly the light-induced phosphorylation and of counteracting the inhibitory effect of atebrin. The evidence from these three experiments thus indicates that flavin is involved in light-induced phosphorylation in *R. rubrum*. In Fig. 4 it is demonstrated that atebrin also strongly inhibited the chloroplast system, regardless of whether phosphorylation had been initiated by addition of

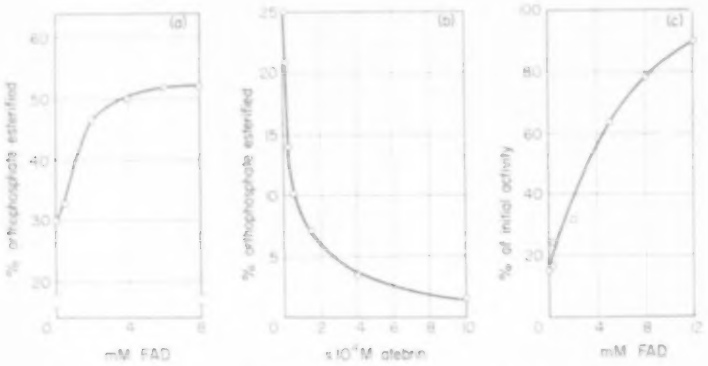


FIG. 3. Evidence for the participation of flavin in light-induced phosphorylation of chromatophores. (a) Stimulation of light-induced phosphorylation by FAD. (b) Inhibition of light-induced phosphorylation by atebrin. (c) Reversal of the inhibitory effect of atebrin on light-induced phosphorylation by FAD. The tubes contained 2×10^{-4} M atebrin. The value of 100 for "per cent of initial activity" corresponds to the value obtained in the absence of atebrin. Experimental details as in Baltscheffsky-[7]. Partially reproduced from *Biochim. Biophys. Acta*[72].

flavin mononucleotide, flavinadenine dinucleotide, menadione, ascorbate or phenazine methosulphate. This indicates that an endogenous flavoprotein must be involved. This possibly is the FAD-containing TPNH-specific diaphorase which has been isolated from chloroplasts by Avron and Jagendorf[66] and can reduce both flavin mononucleotide and menadione. In the chloroplast system no release of the inhibition induced by atebrin was obtained with FAD. On the contrary, it was observed that 2 mM FAD completely inhibited light-induced phosphorylation.[61]

In Fig. 5 it is shown that antimycin A and HOQNO in similar concentrations as those necessary to block mitochondrial respiration[67, 68] gave an inhibition of the bacterial light-induced formation of ATP, whereas in order to obtain inhibition in plant chloroplasts, a roughly thousandfold greater concentration of these inhibitors must be added. Thus, whereas a component of similar nature appears to be acted upon in mitochondria and in chromatophores, the nature of the action of these inhibitors on the chloroplast system is clearly different in some way. From the laboratory of Green [69] evidence has been reported that antimycin A may act upon coenzyme Q in mitochondria. It is interesting in connexion with the inhibitor studies just reported

that coenzyme Q also has been found in *Rhodospirillum rubrum*[70] and that "plastoquinone", a similar but not identical quinone is concentrated in chloroplasts.[61]

The inhibitor studies reported here have supplied evidence for a role of endogenous flavin in light-induced phosphorylation, and flavin is included in the tentative scheme given for the electron transport of this process both in chromatophores and in chloroplasts. As is seen from the schemes, endogenous flavin is assumed to participate in the

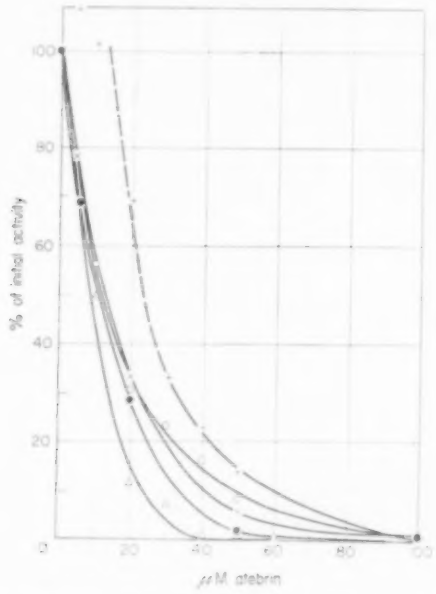


FIG. 4. Effect of atebrin on light-induced phosphorylation in chloroplasts. The following stimulating agents were used: × = FMN, ○ = FAD, △ = phenazine methosulphate, ● = menadione and + = ascorbate. The rate of phosphorylation in the ascorbate series was extremely low. With the exception of the FAD and phenazine methosulphate series all tubes contained 30 μmoles of ascorbate. Experimental details as in Baltscheffsky[61]. Partially reproduced from *Acta Chem. Scand.*[61]

electron transport whichever of the stimulatory agents menadione, flavin mononucleotide or phenazine methosulphate, is used.

Chromatophores

→ flavoprotein → *b*-type cytochrome → coenzyme Q → cytochrome c_2 →

Chloroplasts

→ TPN → flavoprotein → | → ?

menadione
flavin mononucleotide
phenazine methosulphate

In support for the position of flavin in chromatophores in the same chain as the other components may be mentioned the demonstration that the flavinadenine dinucleotide-stimulated part of light-induced phosphorylation was inhibited by low concentrations

of antimycin A and HOQNO.[72] Further support for the position of flavin comes from the finding that phenazine methosulphate, which provides a by-pass for electrons around the site where antimycin A and HOQNO inhibit the light-induced phosphorylation in bacteria[41, 73] has been shown to be an acceptor of electrons from flavoenzyme[74, 75] in a mitochondrial system.

If menadione, flavin mononucleotide and phenazine methosulphate act as stimulating agents for light-induced phosphorylation of isolated chloroplasts by providing a by-pass around some blocked physiological step in the electron transport sequence, it is possible that several of the electron carriers which have been found in chloroplasts

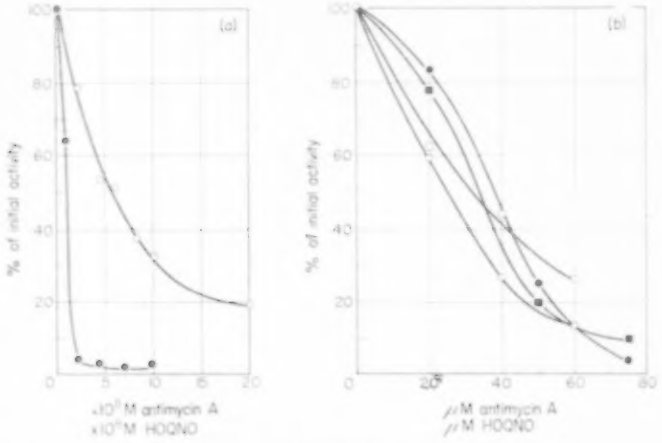


FIG. 5. Effects of antimycin A and HOQNO on light-induced phosphorylation in chromatophores and chloroplasts. (a) Chromatophores: ● = antimycin A, ○ = HOQNO. (b) Chloroplasts. The following stimulating agents were used: ○ and ● = FMN, □ and ■ = phenazine methosulphate. ● and ■ = antimycin A, ○ and □ = HOQNO. Experimental details as in Baltscheffsky[61]. Partially reproduced from *Acta Chem. Scand.*[61]

may be situated in the by-passed portion of the electron transport chain. As an example of a possible similarity between the electron transport in chloroplasts and chromatophores (see above) it may be emphasized that chloroplasts also contain a *b*-type cytochrome (cytochrome b_6[76]), the previously mentioned coenzyme Q-like quinone (plastoquinone[71]) and a spectrally *c*-type cytochrome (cytochrome *f*[77]). One may speculate that an arrangement of individual electron carriers in chloroplasts similar to that suggested above for chromatophores may be possible. From the little information yet available, the redox potentials of the cytochromes[76] would agree with such a sequence; however kinetic data obtained by Chance and Sager[78] do not support it.

Clearly, the mechanisms for electron transport and for formation of ATP in different systems still offer the investigator challenging problems. It is hoped that this presentation has given an impression that interesting similarities have already been found between the different systems for electron transport phosphorylation, and that possibly more similarities are only waiting to be demonstrated. The fact that organelles as different as animal mitochondria, plant chloroplasts, and bacterial chromatophores

have been shown to exhibit several common denominators with respect to their ATP-generating function indicates the usefulness of applying a comparative biochemical approach in this area. Although certain differences have been revealed, and others may be anticipated, it appears fruitful at present to base any working hypothesis for the investigation of electron transport phosphorylation in various sub-cellular systems on the established occurrence of important similarities.

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Biochemical Pharmacology, 1961, Vol. 5, pp. 381–388. Pergamon Press Ltd., Printed in Great Britain.

LIPOPROTEINS AND ELECTRON TRANSPORT

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Abstract—Mitochondrial enzymes are arranged according to a specific pattern. Lipids have been implicated as important components in mitochondrial organization. Some properties of three isolated mitochondrial lipoproteins, the coenzyme Q-lipoprotein, the cytochrome c_1-lipoprotein and the lipoflavoprotein are reviewed, and their role in the function of the electron transport system is discussed.

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OUR current concepts of mitochondrial structure are twofold: the one is based on evidence obtained with the electron microscope, the other on biochemical evidence. It is now very firmly established that mitochondria carry out a great number of fundamental biochemical reactions, the proper functioning of which is essential to all life. Thus a great number of reactions take place in mitochondria, and the appropriate enzymes catalysing these reactions are located within them. The question arises whether these enzymes are randomly distributed in the mitochondrion, or whether they are arranged according to some pattern. The available evidence strongly favours the latter view of a rigid and meaningful structural organization of the mitochondrial enzymes.

Let us not consider this evidence in detail, since it has been discussed elsewhere[1] and earlier at this Symposium[2], but rather move on to some particular aspects of mitochondrial organization, which have arisen in connexion with studies on mitochondrial lipids. It is well known that in addition to a number of redox components, the flavoproteins and the cytochromes, the mitochondria and particles derived from them contain high proportions of lipid material. The electron transport system, when isolated in the form of the ETP_H-particle,[3] which oxidizes DPNH* and succinate by molecular oxygen and concomitantly esterifies phosphate to form ATP, contains about 30 per cent of lipid.

Coenzyme Q

Among the mitochondrial lipids there are specific components that may take part in oxidation–reduction reactions. One such, namely coenzyme Q[4–6] has recently been characterized and studied in detail, and found to be an essential component of the system bringing about oxidative phosphorylation. Coenzyme Q is a tetrasubstituted benzoquinone with two methoxy groups, one methyl group and a long chain consisting of isoprene units as the substituents. The number of isoprene units in the long chain varies from one species to another: mammalian coenzyme Q contains ten isoprene units. The absolute requirement for coenzyme Q in the succinoxidase system and its

* The following abbreviations are used: ATP, adenosine triphosphate; CoQ, coenzyme Q; $CoQH_2$, dihydrocoenzyme Q; cyt. *c*, cytochrome *c*; cyt. c_1, cytochrome c_1; DPNH, reduced diphosphopyridine nucleotide; ETP and ETP_H, the electron transport particle; FAD, flavin adenine dinucleotide; SDC, the succinic dehydrogenase complex.

specificity has been beautifully demonstrated by Lester and Fleischer[7]. Extraction of ETP-particles with organic solvents, such as *iso*octane or acetone abolishes their succinoxidase activity. Coenzyme Q fully restores this activity. That the restored activity closely resembles the activity originally present is shown by the fact that it is completely inhibited by antimycin A. The specificity for coenzyme Q in this system is high; a number of other compounds reported to restore the activity of solvent extracted succinoxidase particles, including some simple derivatives of coenzyme Q do not restore any activity at all.[7]

That coenzyme Q is an equally important link in the electron transport chain, such as the flavins and cytochromes, has been conclusively shown by Hatefi[8]. Thus mitochondrion-bound coenzyme Q shows changes corresponding in its oxidation–reduction levels to those of these other components, and it conforms very nicely with the different steady states given by Chance and Williams[9]. Its involvement in electron transport and oxidative phosphorylation in intact mitochondria seems well established.

Lipoproteins

Recently a number of lipoproteins have been isolated from mitochondria. This advance has thrown much light on the structure and function of the electron transport system. Each of these lipoproteins contains one of the principal components of the electron transport system and this fact, of course, makes them especially interesting from the standpoint both of the structure of the system and of its function.

The isolation of these lipoproteins from mitochondrial suspensions has been made possible by the use of a number of reagents, which readily interact with lipid materials. The successes obtained in the fragmentation of mitochondria by the use of *iso*butyl and amyl alcohols[10] and the salts of bile acids,[11] encouraged investigations on their further effect on submitochondrial fragments. The fraction most thoroughly studied in this respect is the succinic dehydrogenase complex (SDC).[10] Upon treatment with high concentrations of cholate and nearly saturating KCl, the SDC was fragmented into a number of discrete units, with different enzymatic and chemical characteristics.[12] During this fragmentation a lipoprotein was liberated, and it could be separated from the enzymatically active fragments by dialysis and ammonium sulphate fractionation. The combined use of alcohols, bile acids and high salt concentrations has proved to be effective in splitting submitochondrial particles with liberation of two different lipoproteins from them.[13] Furthermore, particles obtained from SDC through the use of cholate and salt can be extracted with solvents such as *iso*octane. Upon lyophilization the *iso*octane extract yields a water-soluble lipoprotein with a strong diaphorase activity.[14] Thus the use of lipid solvents and solubilizing agents has led to the isolation of three different lipoproteins from mitochondria. It is quite conceivable that the use of similar methods may in the future permit the isolation of other lipoproteins from various tissues.

Coenzyme Q–lipoprotein

The first mitochondrial lipoprotein to be isolated was the coenzyme Q–lipoprotein. It can be prepared from the succinic dehydrogenase complex (SDC) by either of two different procedures[15] and is stable upon storage at 4 °C but cannot be frozen. The coenzyme Q–lipoprotein contains large amounts of coenzyme Q; in fact a very large

proportion of the total coenzyme Q in the original succinic dehydrogenase complex (SDC) is recovered in the lipoprotein.

The coenzyme Q–lipoprotein has been studied in considerable detail, and it exhibits many interesting properties, which give it a key position in the electron transport mechanism and possibly also in oxidative phosphorylation. The first role assigned to the lipoprotein was that of a link between the succinic and DPNH dehydrogenase systems in SDC.[12] This interpretation was based on the observation that these two dehydrogenases were separated from each other during the isolation of the lipoprotein. It is now quite clear, however, that this cannot be the only, or even the main, function of the lipoprotein.

A very interesting property of the coenzyme Q–lipoprotein is its ability to take up and bind substances from the surrounding medium. In this ability the lipoprotein shows considerable specificity, as is shown in Table 1.[15] Coenzyme Q is taken up in

TABLE 1. UPTAKE OF SOME COMPOUNDS BY THE COENZYME Q LIPOPROTEIN

Compound	Uptake (μmoles/mg protein)
Coenzyme Q	0·50*
α-tocopherol	0·10
Vitamin A	0·04
Vitamin K_1	0·012
Cyt. *c* (mammalian)	0·0173
Cyt. *c* (*Azotobacter*)	0

* The lipoprotein, as isolated contains 0·15 μmoles coenzyme Q per mg protein.[15]

large amounts, whereas the three other lipid soluble compounds, α-tocopherol, vitamin A and vitamin K, are bound to a much smaller extent. The affinity for CoQ is thus much higher and gives further support to the concept that CoQ is the natural redox component of the lipoprotein.

Cytochrome *c* is also bound to a considerable extent. Here a very remarkable specificity for mammalian cyt. *c* is evident, as cytochrome *c* from *Azotobacter* is not bound. When cyt. *c* is bound by the lipoprotein it exhibits changes in properties, and can now be re-extracted from the lipoprotein with heptane. A similar lipid–cytochrome *c* can also be prepared by the use of particles such as ETP, as Widmer and Crane[16] have shown, since these particles contain the lipoprotein. The ability of the lipoprotein to bind CoQ and cyt. *c* will be seen to be of importance later on, when the functional role of the CoQ lipoprotein is evaluated.

Enzyme systems requiring the coenzyme Q–lipoprotein

A number of enzyme systems requiring the presence of the CoQ–lipoprotein for their proper function have been prepared. As a matter of fact, the *iso*octane extracted ETP is one such system.[4] Its succinoxidase activity is restored by the addition of the CoQ–lipoprotein and the amount of CoQ needed in the form of the lipoprotein is indeed much smaller than when free CoQ is added.

Coenzyme Q oxidase

An enzyme isolated from mitochondria, called CoQ oxidase,[17] also shows a requirement for the lipoprotein (Table 2). This enzyme, which contains only cytochrome *a*

of the cytochromes, catalyses the oxidation of dihydrocoenzyme Q by molecular oxygen. There is an absolute requirement for added cyt. *c*, and a very strong requirement for the lipoprotein. These two components can in part be replaced by the earlier mentioned lipid cytochrome *c*. Dihydrocoenzyme Q is thus oxidized only in presence of cyt. *c*, and especially its lipid soluble form or lipoprotein bound cyt. *c*. Therefore we must assume that cyt. *c* lies between coenzyme Q and cytochrome *a* in the oxidation chain.

Cytochrome c *oxidase*

Another very similar enzyme, cytochrome *c* oxidase, has also been isolated from mitochondria.[18] For maximum activity this enzyme shows a similar requirement

TABLE 2. THE REQUIREMENT FOR COENZYME Q LIPOPROTEIN AND CYTOCHROME *c* IN THE COENZYME Q OXIDASE AND CYTOCHROME *c* OXIDASE SYSTEMS

Conditions	CoQ oxidase	Cyt. *c* oxidase
(1) Enzyme alone	0*	7·12*
+ lipoprotein	0	21·36
+ cyt. *c*	5·7	—
+ cyt. *c* + lipoprotein	44·7	—
(2) Enzyme alone	0	10·68
+ lipoprotein	—	35
+ lipoprotein + cyt. *c*	67·5	—
+ lipid cyt. *c*	31·0	15·7

* The figures giving the rates of oxidation in the two systems should not be compared with each other, since the methods used for assay of the different enzymatic activities do not give comparable results. For details, see the original papers by Hatefi[17, 18].

TABLE. 3. COMPARISON OF CoQ OXIDASE AND CYT. *c* OXIDASE

Substrate	Rate of oxidation by	
	CoQ oxidase	Cyt. *c* oxidase
$CoQH_2$	rapid	slow
Ferrocyt. *c*	rapid	rapid

for the lipoprotein (see Table 2). Here again, lipid cytochrome *c* has an effect, but much smaller than in the previous case.

These two enzymes, CoQ oxidase and cyt. *c* oxidase, which are both isolated from beef heart mitochondria, thus show rather interesting properties with respect to the lipoprotein. Neither enzyme is capable of oxidizing its substrate maximally without the lipoprotein and both contain only cytochrome *a* of the haems. In Table 3, the two enzymes are compared in respect of their substrate specificity. Coenzyme Q oxidase rapidly oxidizes both dihydro-CoQ and ferrocytochrome *c*, whereas cyt. *c* oxidase is unable to oxidise dihydro-CoQ at a speed comparable to the rate of ferrocytochrome *c* oxidation. It therefore seems possible that cytochrome oxidase is a part of the CoQ oxidase. The latter can be stripped of an enzyme necessary for transferring electrons from dihydro-CoQ to cytochrome *c*. From all the data on the two

oxidases and their requirements for maximal activity, it is possible to place cyt. *c* with considerable confidence between coenzyme Q and cytochrome oxidase in the oxidation sequence.

So far it has not been possible to decide with any certainty which is the member of the electron transport chain from which CoQ receives electrons. An experiment by Hatefi[17] seems to rule out cytochrome c_1. Cytochrome oxidase supplemented with catalytic amounts of cyt. *c*, rapidly oxidises purified ferrocytochrome c_1. The rate of oxidation is almost the same as that obtained for ferrocytochrome *c* under the same conditions. Coenzyme Q does not seem to be implicated in this oxidation of ferrocytochrome c_1, and one may therefore conclude that cyt. c_1 can be oxidized through the mediation of cyt. *c* only.

Although much misunderstanding may arise from the presentation of pictures describing electron transport pathways, the data discussed above have been summarized in Fig. 1, which is based mainly on a discussion by Hatefi[17]. The encircled

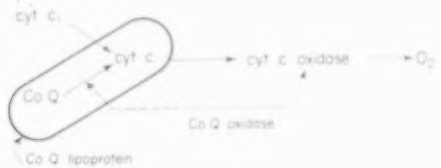

FIG. 1. The flow of electrons from cytochrome c_1 and coenzyme Q to oxygen.

area represents the coenzyme Q lipoprotein, which is shown to contain both CoQ and cyt. *c*. CoQ is there in the first place, when the lipoprotein is isolated and, as was shown, cyt. *c* is readily bound by the lipoprotein. The oxidation of added external $CoQH_2$ starts by equilibrium with the CoQ in the lipoprotein. Electrons are then transferred to cyt. *c* by an enzyme present in the CoQ oxidase , as indicated. Cyt. *c* is then oxidized by the cytochrome oxidase, which transfers the electrons to molecular oxygen. The oxidation pathway of cyt. c_1 is also shown, and passes again through cyt. *c*. Cyt. *c* thus seems to be at the cross-roads, collecting electrons from at least two directions and being oxidized by a single oxidase.

What there should be on the left side of this diagram is not fully known. So far it seems that there is insufficient evidence as to which components of the electron transport system directly precede CoQ and cyt. c_1. There are, of course, not many components left, but their arrangement still presents a puzzle.

Cytochrome c_1 *lipoprotein*

In addition to the CoQ lipoprotein, a lipoprotein containing or associated with cytochrome c_1 has been isolated.[13] In the first phase of the isolation of cyt. c_1 from SDC, this cytochrome is obtained in a form containing about 50 per cent lipid. Table 4 shows some data on this complex and the purified cyt. c_1. The fact that the lipoprotein–cyt. c_1 does not contain any CoQ and has a much lower phospholipid content than the CoQ–lipoprotein, makes it probable that the lipoprotein associated with cyt. c_1 is not identical with the CoQ–lipoprotein. Furthermore, the latter could be isolated in good yield by the same procedure as the cyt. c_1 lipoprotein but appears

during the isolation procedure in an entirely different fraction. The formidable difficulties of obtaining any sizeable amounts of the cyt. c_1-lipoprotein have so far made a more detailed study of its properties impossible.

Flavolipoprotein

The work on the isolation and characterization of mitochondrial components in their natural form has met with extremely interesting success in the isolation by

TABLE 4. THE TWO FORMS OF CYTOCHROME c_1 ISOLATED[13]

	Cytochrome c_1 lipoprotein complex	Free cytochrome c_1
Haem (mμmoles/mg protein)	6·05	14·7
Lipid (% of dry wt.)	52	0
Coenzyme Q	none	—
Phospholipid (% of total)	80	—

Ziegler *et al.*[14] of a flavolipoprotein showing a very high diaphorase activity.

One of the observations that led to this discovery was that the activity of ETP, when expressed as DPNH oxidized per unit of flavin, was very much higher than in the purified DPNH–cyt. *c* reductase preparations described by Mahler *et al.*[19] and by de Bernard[20]. This fact is shown in Table 5. The recently isolated lipoflavoprotein

TABLE 5. DIAPHORASE ACTIVITY PER UNIT OF ACID EXTRACTABLE FLAVIN

ETP	25–35 μmoles DPNH/mμmole flavin
Lipoflavoprotein	32 μmoles DPNH/mμmole flavin
Cyt. *c* reductase	2·2 μmoles DPNH/mμmole flavin

Data from Ziegler *et al.*[14]

shows an activity similar to that of ETP. All these activities were measured with ferricyanide as electron acceptor.

A number of different flavoproteins with diaphorase activity have been isolated from mitochondria.[16] With respect to flavin content those mentioned above, and the Straub[21] diaphorase are about equally purified. In the cytochrome reductase, none of the flavin, which is easily and quantitatively extracted by acid, can be assayed as FAD in the D-amino acid oxidase system.[19, 20] In the others, the Straub[21] diaphorase and the lipoflavoprotein,[14] all of the flavin is accounted for by FAD. There are also considerable differences in the activities of the different flavoproteins when different electron acceptors are used. The lipoflavoprotein shows the highest activity with ferricyanide, whereas the lipid-free flavoproteins are practically inactive with ferricyanide but show a very high activity with methylene blue as electron acceptor. The similarity in properties between the classical Straub diaphorase and a flavoprotein obtained from the lipoflavoprotein by complete removal of the lipid is most striking, as is the change in properties when this conversion takes place.

The question about the relation between the Straub[21] diaphorase and the cyt. *c* reductase[19, 20] now seems to be settled in the light of the evidence provided by the extraction of the lipoflavoprotein. Table 6 shows that the extraction method used for the liberation of the cyt. *c* reductase is capable of converting its flavin into a form which does not function as FAD in the D-amino acid oxidase assay, while the other extraction method does not lead to such a conversion. The form of flavin encountered in the cyt. *c* reductase is thus an artifact of preparation, and one may therefore ask

TABLE 6. RECOVERY OF FLAVIN IN THE PREPARATION OF DIAPHORASES FROM ETPH BY TWO DIFFERENT EXTRACTION METHODS

Enzyme	Total acid extractable flavin (mμmoles/mg)	(% recovery)	FAD %
ETP_H	0·2	—	100
Lipoflavoprotein (first extract)	0·9	80	98
Cyt. *c* reductase (first extract)	9·0	80	2–5

From Ziegler *et al.*[14]

whether the cyt. *c* reductase activity might not also be an artifact, since it is not present in the lipoflavoprotein or the Straub diaphorase, which both contain FAD only. Such a situation necessitates a re-evaluation of the concepts of electron transport pathways in mitochondria.

CONCLUDING REMARKS

The lipoproteins which I have described in the foregoing show many interesting properties. Their role in electron transport is not yet fully understood, but the observation, that three of the main components of the terminal oxidation sequence, diaphorase, cyt. c_1 and CoQ are closely associated with lipoproteins is very suggestive.

Apart from the role in electron transport which must be attributed to the lipoproteins, a role in the mechanism of oxidative phosphorylation has to be envisaged. Recently Clark *et al.*[22] (and other authors) have indicated that compounds having the quinone structure may play a decisive role in phosphorylation. Furthermore, Löw[23] has produced evidence of the direct participation of mitochondrial flavins in the ATPase and ATP–inorganic phosphate exchange reactions, which have been thought to be "shadows" of oxidative phosphorylation.

This evidence must now be considered in the light of the investigations of Grabe[24], which indicate that phosphorylative processes may be favoured by a milieu where access of water to the phosphate compounds, presumably formed as intermediates, is restricted. The lipoproteins could obviously provide such a water-free environment, and the fact that quinone-like compounds (CoQ and flavins) have been found associated with lipoproteins, makes the assumption that the lipoproteins play a role in oxidative phosphorylation even more attractive.

Acknowledgement—I wish to express my most sincere gratitude to Dr. David Green, in whose laboratory I had an excellent opportunity to become familiar with these exciting problems, and who also encouraged me to discuss them at this meeting.

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Biochemical Pharmacology, 1961, Vol. 5, pp. 389–391. Pergamon Press Ltd., Printed in Great Britain.

THE ROLE OF NORADRENALINE AND 5-HYDROXYTRYPTAMINE IN THE CENTRAL ACTION OF RAUWOLFIA ALKALOIDS AND BENZOQUINOLIZINE DERIVATIVES

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Abstract—Sedative-like Rauwolfia alkaloids lower the content of 5-hydroxytryptamine and noradrenaline in brain and other tissues. Benzoquinolizine derivatives lacking an indole group have a similar effect upon these amines. A difference in these two classes of compounds was found when the latter substances produced a short sedative effect and brief depression of the amines. In general the results suggest a causal relation between the pharmacological action of the two types of drugs upon the central nervous system and monoamine metabolism. Studies with other benzoquinolizine compounds show a differential effect upon the two amines so that noradrenaline depletion may cause sedation. Reserpine-like drugs do not have identical effects upon brain and peripheral amines. It is possible that tranquilizing effects are exerted through depletion of noradrenaline or its precursors or of other substances yet unknown.

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FOLLOWING the work of Pletscher *et al.*[1], Carlsson and Hillarp[2] and Holzbauer and Vogt[3] several reports have been published, which show that reserpine lowers the content of 5-hydroxytryptamine (5-HT) and catechol amines, particularly noradrenaline (NA), in the brain and various other tissues. Among Rauwolfia alkaloids only those with sedative action (reserpine, raunescine, deserpidine, rescinnamine) produce depression of the two amines.[4, 5] Pletscher *et al.*[6, 7] have found a second group of substances, which, like Rauwolfia alkaloids, cause sedation as well as NA and 5-HT depletion in the brain. These are synthetic benzoquinolizine derivatives having no indole group.

After Rauwolfia administration the content of NA and 5-HT diminishes gradually during several hours and the sedative action also becomes clear only after a considerable latent period. Reserpine, apart from small traces, is metabolized in most tissues long before the pharmacological effects have been fully developed.[8, 9] Benzoquinolizines, on the other hand, produce a comparatively short-lasting depletion of these brain amines and the sedative effect is also short-lived.[7] Based on these findings, among others, it has been postulated that a causal connexion exists between the pharmacological action of the afore-mentioned drugs on the central nervous system and their influence on monoamine metabolism.

According to the theory of Brodie and co-workers[10, 11] 5-HT exists in the tissues in "bound" form and only minute quantities are in "free" state. If the pharmacological activity is due to the unbound fraction the sedative action of Rauwolfia could be produced by the free 5-HT liberated from the cellular binding sites. A relative increase in this free amine would then be more important than the decrease of the total 5-HT present. It is difficult to explain, for instance, why Rauwolfia alkaloids

produce stimulation instead of sedation in animals pretreated with monoamine oxidase inhibitors, like iproniazid.[11] After iproniazid treatment these alkaloids can even increase the total 5-HT content in the brain[12] and at the same time probably also elevate the amount of unbound amine. More evidence for the role of 5-HT has been presented,[13] but many of the recent results indicate that the pharmacological effects of these compounds on the central nervous system may possibly be related to the depression of NA but probably not to that of 5-HT.

Carlsson *et al.*[14] have shown that 3 : 4-dihydroxyphenylalanine (DOPA), the precursor of NA, when given to reserpinized mice and rabbits, will restore normal activity presumably by restoring the central catechol amine. The precursor of 5-HT, 5-hydroxytryptophane, however, was unable to antagonize the tranquilizing effect of reserpine. Essentially similar results have been obtained while using monkeys treated with deserpidine.[15] Carlsson *et al.*[16] have also found that, like NA, the precursor of it, 3-hydroxytyramine (Dopamine) is made to disappear almost completely from the brain by reserpine.

It has been demonstrated by Brodie *et al.*[10] while using fluorometric methods that reserpine depletes 5-HT and NA in the rabbit brain stem to the same degree and at the same rate, the two curves being superimposable. With biological methods we found, however, that after raunescine in rats the depletion of NA in the brain was much more pronounced than that of 5-HT.[17, 18] This happened also after a suitable dose of reserpine, although not as consistently.[17] There was a clear sedative action after a dose of raunescine which was able to lower the NA but not the 5-HT content in the brain.

This selective action on brain monoamines has been demonstrated by Pletscher *et al.*[19] by using two benzoquinolizine derivatives. These were found to affect the brain content of 5-HT in mice almost equally but the brain content of NA differently. One of the compounds was able to release more NA than the other and only the NA-releasing compound had a marked sedative effect.

It is of interest that some new drugs, which have some of the actions of reserpine, cause depression of the amines mainly in that part of the organism where their site of action is most obvious, i.e. centrally or peripherally. Ro 1–9569 (tetrabenazine) a benzoquinolizine derivative, releases NA and 5-HT in the brain but does not appreciably affect NA at peripheral nerve endings.[13,20] It also elicits reserpine-like central effects but has no marked action on the blood pressure.[20] From synthetic reserpine analogues SU 5171 exhibits sedative activity and depletes brain 5-HT in low doses, but does not depress the blood pressure. On the other hand, another analogue, SU 3118 (syrosingopine) is effective in decreasing the blood pressure and releasing peripheral NA, but considerable higher doses of this compound are necessary to produce sedation and depletion of brain NA and 5-HT.[21–23]

In the periphery the reserpine-like drugs probably produce their typical effects by making cells unable to bind NA. Which of the two amines is more important in the mechanism of the central action of these drugs is not clear. It is also possible that other substances present in the brain cells will still enter the picture.

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THE SUBCELLULAR LOCALIZATION OF TRANSMITTER SUBSTANCES IN THE CENTRAL NERVOUS SYSTEM

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Abstract—The chemical nerve transmitter, acetylcholine, is thought to be contained in vesicles found in nerve endings. In support of this hypothesis nervous tissue was fractionated; acetylcholine and choline acetylase were found to have a similar distribution in sub-cellular fractions. Acetylcholine binding particles were easily separated from mitochondria by use of equilibrium centrifugation in a density gradient. About half of the bound acetylcholine was released from the particles by mechanical agitation, hypo-osmotic media or treatment with cobra venom, while the remaining bound acetylcholine fraction was freed by treatment with organic solvents, acids, supersonic vibrations or a temperature of 35 °C. The two types of bound acetylcholine are explained on the basis of a differential type of storage mechanism, one in solution and the other bound to the matrix of the particles by ionic bonds. The significance of the acetylcholine storage particles is discussed.

TRANSMISSION at nerve endings is being very much discussed at the present time in terms of what may be called the "synaptic vesicle theory" of synaptic transmission.[1] Briefly, it is assumed that chemical transmitters are not freely diffused throughout the axoplasm, but are localized in the inactive nerve terminal, inside small vesicles about 0·05 μ in diameter, which electron microscopic studies have shown to be present in large numbers at almost every type of nerve ending. These synaptic vesicles can be seen in Fig. 1 which is an electron micrograph of a nerve ending in lizard brain. The synaptic vesicles seen in mammalian nerve endings are similar in size and appearance. They are assumed to discharge their quantum of transmitter substance as the result of the arrival of a nerve impulse. A low rate of discharge may occur spontaneously all the time, but if so, the amount of transmitter substance released is sufficient to produce only a local "miniature" depolarization of the post-junctional membrane. The arrival of an impulse in the pre-junctional terminals enormously increases the rate of discharge and at the same time synchronizes it, so that the post-junctional potential reaches the proportions associated with a propagated discharge.

The evidence for the quantized release of transmitter comes mainly from the electrophysiological studies of Katz and co-workers[2] on the motor end plate. Katz observed that even in a resting junction, miniature end plate potentials of more or less uniform size made their appearance in random fashion. The size of these potentials indicated that they were being formed as the result of the release of "quanta" of acetylcholine in amounts of a few thousand molecules at a time. The normal end plate potential was shown to be the summed effect of several hundred quanta (about 300

FIG. 1. Nerve endings in lizard brain. (*a*) Myelinated axon; (*m*) mitochondria; (*sv*) synaptic vesicles; (*st*) thickening of synaptic membranes; (*ncb*) neurone cell body. Electron micrograph prepared by B. B. Boycott, E. G. Gray and R. W. Guillery.

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according to the best estimates[3]) released suddenly over an interval of the order of 1 msec.

The electrophysiological and morphological evidence pointing to the existence of synaptic vesicles charged with transmitter substance is also supported by the biochemical evidence. It has long been known that substances such as acetylcholine exist in nervous tissue in a bound, pharmacologically-inactive form in association with the insoluble particulate fraction of the cell. While in this form they are immune from the destructive action of enzymes such as cholinesterase.

If synaptic vesicles really exist within the nerve cell as a distinct type of subcellular particle, it should be possible to isolate them by means of the same sort of techniques as have been successfully used by Schneider, de Duve, Blaschko and others for isolating nuclei, mitochondria, microsomes, lysosomes and chromaffin granules. Their successful isolation and a demonstration that they contain transmitter substance would not only be important supporting evidence for the synaptic vesicle theory; it would also, by enabling the biochemical make-up and properties of the particles to be studied *in vitro*, throw light on the nature of the transmission process itself, and the mode of action of drugs and toxins suspected of interacting with this process.

Work on these lines is being carried out at the Institute of Animal Physiology, Babraham, by Dr. C. O. Hebb and myself[4, 5]. We began by showing that bound acetylcholine and choline acetylase, the enzyme which synthesizes it, have a similar distribution in subcellular fractions prepared from homogenates of brain in iso-osmotic sucrose. The fractions were prepared by differential centrifugation and the greater part was recovered in the so-called mitochondrial fraction. We then showed that the particles containing bound acetylcholine could be separated from mitochondria, using equilibrium centrifugation in a density gradient. The simplified procedure now used[5] is as follows. The original homogenate of brain in 0·32 M sucrose is separated by centrifugation in integrated fields of 10^4 and 8×10^5 *g* min into a fraction comprised mainly of nuclei and cell debris, a crude mitochondrial fraction, and a cytoplasmic fraction consisting of microsomes suspended in the soluble constituents of cytoplasm diluted with sucrose. The crude mitochondrial fraction is then layered on top of a density gradient consisting of equal volumes of 1·2 M and 0·8 M sucrose, and the whole centrifuged at 39,000 rev/min for 1 hr in the SW39 head of the Spinco model L preparative ultracentrifuge. At the end of the run, the tubes have the appearance shown in Fig. 2. The crude mitochondrial fraction has separated into three well defined fractions. The first consists of particles less dense than 0·8 M sucrose (*A*-layer) which are visible under the phase contrast microscope as small particles of diameter 2–5 μ (Fig. 3(1)). The second (*B*-layer), intermediate in density between 0·8 and 1·2 M sucrose, consists of very small particles below the limit of resolution of the light microscope. The third (*C*-layer), denser than 1·2 M sucrose, consists of typical small rod-shaped mitochondria together with smaller particles.

Fig. 4 shows the distribution of bound acetylcholine and succinic dehydrogenase, a mitochondrial marker, in the various fractions. In the primary fractions, bound acetylcholine and succinic dehydrogenase have a similar distribution, about 70 per cent of each being recovered in the crude mitochondrial fraction. This parallel distribution shows that the bound acetylcholine content of the nuclear and microsomal fractions can be accounted for by the contamination of these fractions by particles broadly similar in sedimentation properties to mitochondria. By contrast, the distribution

of bound acetylcholine in the sub-fractions of the crude mitochondrial fraction is quite different from that of succinic dehydrogenase. The bound acetylcholine is seen to be associated mainly with the *B*-layer and the succinic dehydrogenase with the *C*-layer.

Under the electron microscope (Fig. 3(3)), the *C*-layer, as would be expected from its succinic dehydrogenase content, consists mainly of particles (*m*) having the typical internal structure of mitochondria. Compared with mitochondria fixed *in situ*, these appear vesicated, some have lost their outer membranes, or their cristae have shrunk and have been drawn to one pole. Many, however, are recognizable as reasonably intact mitochondria.

The *B*-layer (Fig. 3(2)) presents quite a different appearance. Here no mitochondria are visible. Instead we have large numbers of very small particles (*a*) appearing in section as simple vesicles (*v*) and varying in size from about 0·02 μ to 0·28 μ, with a

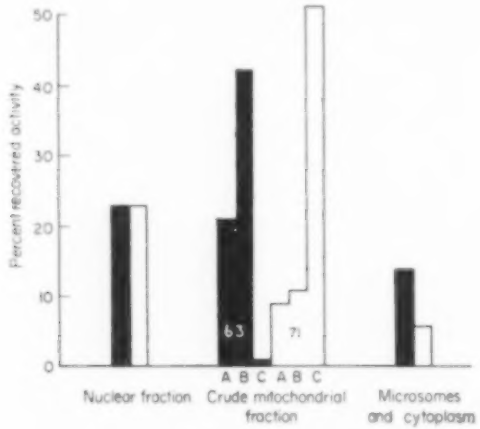

FIG. 4. Distribution of bound acetylcholine (black blocks) and succinic dehydrogenase (white blocks) in subcellular fractions of guinea pig brain.

median of 0·065 μ. Over 60 per cent of these particle fall within the size range 0·02 to 0·08 μ given by Palay[6] for synaptic vesicles. The differences in size range of the two populations of particles is shown in Fig. 5. Here the percentages of particles possessing a given size or less are plotted on a linear scale of probits as ordinates and the size on a logarithmic scale as abscissae. The particles of the *B*-layer (open circles) are seen to have a log-normal size distribution; those of the *C*-layer (solid circles) are also seen to be lognormally distributed except at the lower end of the range. However, if the *C*-layer in this experiment is assumed to be 10 per cent contaminated with particles of the *B*-layer, the corrected distribution (crosses) follows a lognormal distribution throughout its range. As shown in Fig. 3(3), the *C*-fraction contains some particles morphologically identical with those of the *B*-layer.

The characteristics of the *B*-layer, the size range of its particles, their simple vesicular structure and their high content of a transmitter substance, are all consistent with the idea that the *B*-layer represents a crude preparation of isolated synaptic vesicles.

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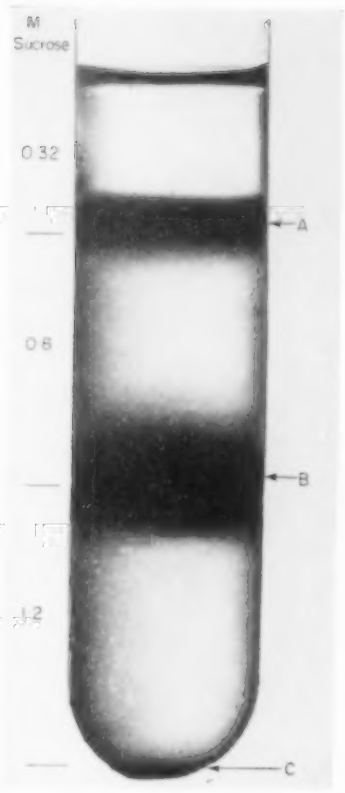

FIG. 2. Photograph of a centrifuge tube after separation of a crude mitochondrial fraction of guinea pig brain into three sub-fractions by equilibrium centrifugation in a density gradient. *A*, particles less dense than 0·8 M sucrose; *B*, particles intermediate in density between 0·8 and 1·2 M sucrose; *C*, particles denser than 1·2 M sucrose.

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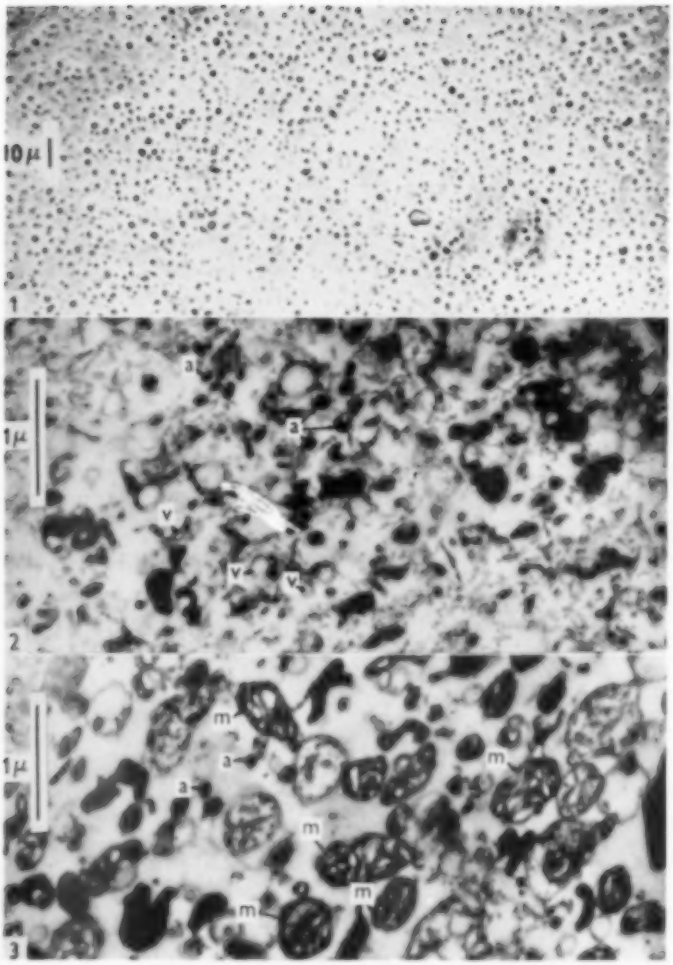

FIG. 3. (1) Phase contrast micrograph of fraction *A* of guinea pig brain. Wet preparation stained with osmium tetroxide. (2) Electron micrograph of thin sections of particles of the *B*-layer fixed and stained with osmium tetroxide and embedded in methylmethacrylate resin. (3) Particles from the *C*-layer similarly treated. The electron micrographs were prepared by Dr. K. M. Smith and Mr. G. J. Hills.

The acetylcholine-containing particles of the *B*-layer are quite stable for a few hours if kept in isotonic media at 0 °C. Amounts of purified cholinesterase capable of destroying an equivalent amount of free acetylcholine in a few seconds have no effect on particle-bound acetylcholine. On the other hand, if warmed to 37 °C or brought to below pH 4·5, all the acetylcholine is quickly released from the particles.

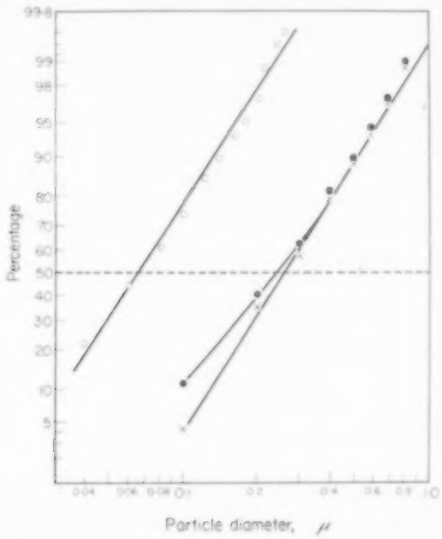

FIG. 5. Distribution of particle size in *B*- and *C*-fractions from guinea pig brain. Ordinates: percentage of total population having indicated diameter or below, plotted on linear scale of probits. Abscissae, particle diameters on logarithmic scale.

A closer study of the conditions bringing about release of acetylcholine support Brodkin and Elliott's[7] finding that about half the acetylcholine is bound in a particularly labile way. Mild disruptive treatments such as freezing and thawing, suspension in hypo-osmotic media, mechanical agitation or treatment with cobra venom release about 50–60 per cent of the total acetylcholine; the remainder requires more drastic treatment such as treatment with organic solvents, acids, supersonic vibrations or incubation at 37 °C for release. These findings are consistent with the idea that part of the acetylcholine is held inside the particle in simple solution and requires only a small permeability change in the particle membrane for release, while the other part is bound more firmly to the matrix of the particle, perhaps by ionic bonds. Experiments with ion exchange resins have suggested that ionic links between the acetylcholine cation and weak acid groups in the particle could, under certain circumstances, be strong enough to account for this latter form and would also account for the effect of pH on the binding.

If the *B*-layer is indeed a crude preparation of synaptic vesicles, we should expect other central nervous transmitters besides acetylcholine to be concentrated there. It was therefore of great interest to us to find that bound 5-hydroxytryptamine is also specifically concentrated in this layer as shown in Fig. 6. Hydroxytryptamine particles of the type described by Baker[8] were not present in brain. It is possible that the *B*-layer

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is a rich storehouse of other pharmacologically active substances and that the presence of a substance there would be *prima facie* evidence for a transmitter action.

Bound hydroxytryptamine is released under much the same conditions as acetylcholine. There seem, however, to be important differences in the mechanism of storage.

The close parallelism between the distribution of acetylcholine and choline acetylase,[4] the "occluded" state of the latter in particles whose structural integrity has not

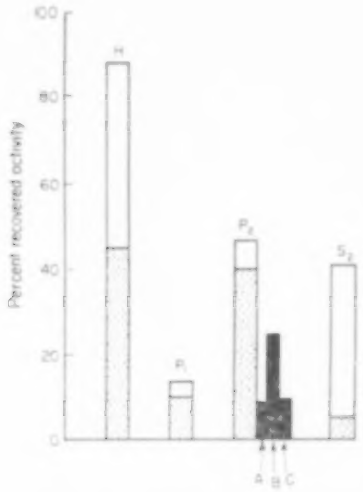

FIG. 6. Bound (dotted blocks) and free (white blocks) hydroxytryptamine in a sucrose homogenate of guinea pig brain (H) and in nuclear (P_1), crude mitochondrial (P_2) and supernatant (S_2) fractions derived from it. It will be seen that most of the free amine in H is recovered in the supernatant fraction while the bound is recovered in the crude mitochondrial fraction. The black blocks show the distribution of total hydroxytryptamine in the sub-fractions of P_2, the highest concentration being found in the *B*-fraction.

been destroyed[9] and the absence of "acetate activating enzyme" in the particles[10] suggest that acetylcholine is synthesized *in situ* from choline and acetylcoenzyme A. By contrast, a non-particulate location has been assigned to the enzyme 5-hydroxytrytophan decarboxylase responsible for the formation of hydroxytryptamine;[11] this implies that the hydroxytryptamine particles do not synthesize hydroxytryptamine *in situ* but that they take it up from the cytoplasm.

There is considerable evidence that adenosine triphosphate (ATP) is involved in some way in the binding of pressor amines to their storage granules. No studies have been made of the distribution of ATP in the brain fractions, though the absence of a typical component of the electron transport chain from the particles of the *B*-layer suggests that they are unable to synthesize it. Some preliminary experiments (Fig. 7) indicate, however, that they can take up hydroxytryptamine from the supernatant and that this process is ATP dependent. In contrast to the findings of Walaczek and Abood[12], reserpine does not appear to affect this process, nor does it deplete the particles of hydroxytryptamine *in vitro*, though it is possible to prepare depleted particles from animals previously treated with reserpine *in vivo*. These findings suggest

that reserpine does not act directly on the storage particles, but indirectly, perhaps by influencing the general level of availability of ATP. Walaczek and Abood's report[13] that reserpine is a decoupling agent in mitochondrial oxidative phosphorylation deserves further investigation in this connexion.

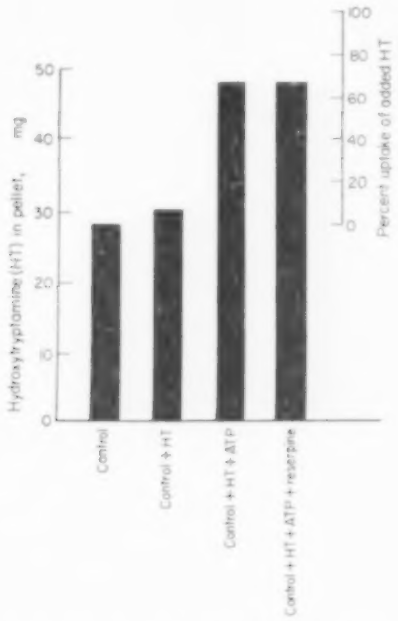

FIG. 7. Uptake of 5-hydroxytryptamine by brain particles in the presence of ATP. The crude mitochondrial (P_2) fraction from guinea pig brain (1 ml, equivalent to 450 mg original brain tissue) was kept at 0 °C for 1 hr alone (control) or with reserpine (25 μg), ATP (10 μmoles) and/or 5-hydroxytryptamine (30 μg free base) in a total volume of 1·48 ml 0·32 M sucrose as indicated. The samples were then centrifuged in an integrated field of 10^6 *g* min, the pellet taken up in 1 ml 0·32 M sucrose, heated for 10 min at pH 4·3 to release bound hydroxytryptamine and the latter assayed on the rat fundus strip[13].

Much more remains to be done in the study of these storage particles from brain, but I hope to have demonstrated how this may be expected to lead to further understanding of the mode of action of central nervous system drugs and the release of transmitter substances.

Acknowledgements—I am most grateful to Dr. E. G. Gray for furnishing the electron micrograph shown in Fig. 1 and to Dr. K. M. Smith and Mr. G. J. Hills for the electron micrographs in Fig. 3.

Note added in proof: Recent work by E. G. Gray and V. P. Whittaker (*J. Physiol. Proc.*, 9th July 1960) using an improved electron microscopic technique has revealed that the smallest particles in the *B* layer are enclosed within thin membranes which become extremely fragile after osmication. The small particles within these larger membranes now closely resemble synaptic vesicles as seen in whole tissue sections. A comparison of the morphology of the the larger particles with that of nerve endings in mammalian cortex indicates that they are in fact pinched-off nerve endings. Occasionally particles are seen containing mitochondria in addition to synaptic vesicles and with a portion of post-synaptic membrane still adhering to their periphery.

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Papers to be published in future publications

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E. Paroli and P. Melchiorri: Inhibitory effects of morphine on metabolism of adrenal and testicular steroids.

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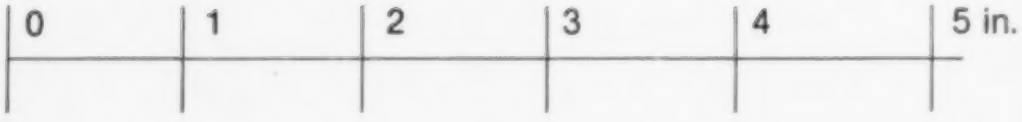

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